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(54) METHOD USING COPEPTIN TO PREDICT ONSET OF PREECLAMPSIA

VEERWENDUNG VON COPEPTIN ZUR VORHERSAGE DES AUSBRECHENS VON PRÄEKLAMPSIE

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Description

BACKGROUND OF THE INVENTION

[0001] Hypertension complicates up to 10% of all pregnancies worldwide. In the United States, preeclampsia affects 5-7% of all pregnancies, approximately 300,000 pregnancies a year. Yet, it disproportionately represents 15% of all maternal-fetal morbidity and mortality. Preeclampsia is known to cause immediate maternal-fetal morbidities such as growth restriction, oligohydramnios, fetal death, maternal seizures, stroke, cerebrovascular hemorrhage, and maternal death (73). Mothers with a history of preeclampsia are at increased risk of future cardiac disease including myocardial infarction and stroke (23, 52, 53). Children born from preeclamptic pregnancies are also at increased risk of stroke (40), epilepsy (91), and metabolic, nutritional and blood disease (90) in later childhood or as an adult. Clearly, preeclampsia has immediate and long term effects on both the fetus and mother. However, its pathogenesis is poorly understood. Consequently, preventative, therapeutic, and curative modalities for preeclampsia are elusive. The only true cure for preeclampsia is the delivery of the fetus and dysfunctional placenta. This delivery is often preterm and contributes to additional morbidity and mortality (73). This fact emphasizes the importance of finding appropriate unifying pathways to be able to treat preeclampsia.

[0002] The neurohypophysial hormone, arginine vasopressin (AVP; FIG. 1), is a known regulator of blood pressure and composition in human and animal models. AVP is a major player in blood pressure control in selected populations including African Americans (4), the elderly (19), and in patients with congestive heart (25) or renal failure (3). This hormone appears to specifically be causative in patients with low-renin hypertension (76), which makes up a larger portion of the human essential hypertensive population (27%) than high-renin hypertension (16%) (49).

[0003] Evidence supports a potential causative role for AVP in the development of preeclampsia. Copeptin is translated together with AVP and is released into the plasma in a 1:1 stoichiometric ratio to AVP (FIG. 1). Because copeptin is much more stable than AVP in the circulation, measurements of this peptide are much more consistent and reliable than direct measures of AVP. Late-trimester plasma copeptin levels have been correlated with the severity of preeclampsia and with associated abnormal placental Doppler velocimetry in humans (21, 101). Yet, the expression pattern of copeptin in early pregnancies is undetermined.

SUMMARY OF THE INVENTION

[0004] The present invention is directed to a method of diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject, the method comprising measuring differences in copeptin levels in a sample collected from a subject during the first trimester of pregnancy compared to a control using an antibody detection assay, wherein the sample is blood, serum, plasma or urine, and wherein an increase in copeptin levels of about 1/4 fold compared to a control is predictive of the occurrence of preeclampsia during the subject's pregnancy.

[0005] In an embodiment of the invention, the method further comprising taking Doppler velocimetry measurements on at least one of the subject's uterine and umbilical arteries.

[0006] In an embodiment of the invention, the sample is urine.

[0007] In an embodiment of the invention, the assay comprises a test strip or an ELISA.

[0008] Also disclosed herein as a first aspect of the disclosure is a kit for diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject includes an antibody detection assay specific for the detection of copeptin in a sample taken from the subject during the first trimester of pregnancy.

[0009] In one embodiment of this aspect of the disclosure, the sample includes at least one of blood, serum, plasma, or urine.

[0010] In a further embodiment of this aspect of the disclosure, the sample is urine.

[0011] In another embodiment of the kit disclosed in this aspect of the disclosure, the assay includes a test strip or an ELISA.

[0012] In a further embodiment of this aspect of the disclosure, an increase in copeptin levels of about 1/4 fold compared to a control is predictive of the occurrence of preeclampsia during the subject's pregnancy.

[0013] In another embodiment of this aspect of the disclosure, the antibody detection assay also detects vasopressin and neurophysin II.

[0014] Also disclosed herein as a second aspect of the disclosure is a method of diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject includes collecting a urine sample from a subject during the first trimester of pregnancy, and measuring differences in copeptin levels compared to a control using an antibody detection assay. In one embodiment of this aspect of the disclosure, the method further includes taking Doppler velocimetry measurements on at least one of the subject's uterine and umbilical arteries.

[0015] Also disclosed herein as a third aspect of the disclosure is a kit for diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject includes an enzyme detection assay specific for the detection of LNPEP in a sample taken from the subject.

[0016] In one embodiment of this aspect of the disclosure, the enzyme detection assay is an antibody detection assay.

[0017] In a further embodiment of this aspect of the disclosure, the enzyme detection assay is an enzymatic activity assay.

[0018] In another embodiment of this aspect of the disclosure, the kit further includes an antibody detection assay specific for copeptin.

[0019] In another embodiment of this aspect of the disclosure, a decrease in LNPEP levels and an increase in copeptin levels of about 1/4 fold compared to a control is predictive of the occurrence of preeclampsia during the subject's pregnancy.

[0020] Also disclosed herein as a fourth aspect of the disclosure is a test device for predicting whether a subject is predisposed to developing preeclampsia includes a substrate including a test assay for detection of a protein product of the vasopressin gene, a sample application area, and a read out area. Upon application of a sample from a subject by a user, the test device provides information to the user indicating whether the subject is pregnant and whether the subject is predisposed to developing preeclampsia.

[0021] In one embodiment of the device of the disclosure, the substrate comprises at least one of plastic, glass, metal, cellulosic material, a polymer, and a cloth, and combinations thereof.

[0022] In another embodiment of the device of the disclosure, the sample application area is fluidly connected to the readout area.

[0023] In a further embodiment of the device of the disclosure, upon application of a sample from a subject by a user to the sample application area, the detection assay may be engaged within the device.

[0024] In another embodiment of the device of the disclosure, the information provided to the user comprises an indicium in the read out area.

[0025] In a further embodiment of the device of the disclosure, the device further includes user instructions for interpreting the information provided by the device.

[0026] In another embodiment of the disclosure, the test device comprises a plurality of test assays.

BRIEF DESCRIPTION OF THE FIGURES

[0027]

FIG. 1 depicts the protein product of the vasopressin (AVP) gene. The signal sequence targets the protein for cellular export. Arginine vasopressin (AVP) is then produced and released in a 1:1 molar ratio with the AVP carrier protein neurophysin II, and with copeptin. While AVP exhibits a very short half-life within the plasma, copeptin is much more stable and is primarily cleared into the urine where it can be detected easily by immuno-based assays (3, 5).

FIG. 2 depicts a test assay contemplated herein. Panel A depicts an unused test assay device, panel B depicts a device that a user has applied a sample to where the device indicates that the sample came from a pregnant woman (positive sign) who does not have an elevated risk for preeclampsia (negative sign), and panel C depicts a device that a user has applied a sample to that indicates that the sample came from a pregnant woman with increased risk of preeclampsia (double positive signs).

FIG. 3. Elevated vasopressin in sRA mice. *A*: arginine vasopressin (AVP) immunoreactivity in the supraoptic (SON, *top* and *middle* rows, from four separate animals) and paraventricular (PVN, *bottom* row, from two separate animals) nuclei in female sRA and control animals. Note the increased numbers of strongly immunoreactive AVP neurons in the retrochiasmatic part of the SON in sRA animals. ON, optic tract; 3V, third ventricle. Bars = 200 μm. *B*: total immunoreactive cell fragments per side, greater than 10 μm in diameter, in four serial sections (spaced 200 μm apart) through the PVN and SON of littermate control and sRA mice (n = 3 females each group). *C*: plasma copeptin levels (n = 4 male + 4 female control, 4 male + 4 female sRA). *D*: urine copeptin concentration, total daily urine volume, and total daily copeptin loss into urine (n = 12 male + 5 female control, 10 male + 7 female sRA). All data are means ± SE. *$P < 0.05$ vs. control.

FIG. 4. Blood pressure responses to vasopressin receptor antagonists. *A*: systolic blood pressure (BP), monitored by tail-cuff, at baseline and with 10 days of chronic subcutaneous infusion (22 ng/h) of the V1A/V2 nonpeptide antagonist conivaptan (n = 2 male + 4 female control, 2 male + 4 female sRA). Hourly telemetric blood pressure (B, MAP) and heart rate (*C*, HR) recordings for 3 days preceding and 18 days during subcutaneous infusion of the nonselective V1A/V2 receptor antagonist conivaptan (22 ng/h) in a female sRA mouse are shown. *D*: spontaneous ambulatory physical activity counts during conivaptan infusion experiment (in *B* and *C*). *E:* systolic BP, monitored by tail-cuff, at baseline and with 10 days of chronic subcutaneous infusion (22 ng/h) of the V2-selective antagonist tolvaptan (n = 4 male + 5 female control, 4 male + 6 female sRA). *F*: hourly average radiotelemetric MAP recordings from (n = 4 female) sRA mice at baseline and after 10 days of subcutaneous tolvaptan infusion (Drug X Time, P = 0.029). All data are means ± SE. *$P < 0.05$ vs. control, †$P < 0.05$ vs. baseline sRA.

FIG. 5. Vascular reactivity of abdominal aorta. *A*: maximum contractile response to 100 mmol/l KCl. *B* and C: relaxation responses to graded doses of acetylcholine and sodium nitroprusside after half-maximal contraction to PGF_{2a}, *D-H:* contractile responses to graded doses of arginine vasopressin, phenylephrine, endothelin-1, angiotensin II, and prostaglandin-F_{2a} (PGF_{2a}) (*n* = 6 male control, 5 male sRA). All data are means ± SE. **P* < 0.05 vs. control.

FIG. 6. Mesenteric artery vascular reactivity. *A*: maximum contractile response to 100 mmol/l KCl. *B:* contractile responses to graded doses of arginine vasopressin, phenylephrine, and endothelin-1 (*n* = 6 male control, 6 male sRA). *C*: external and lumen diameters, wall thickness, media-to-lumen ratio, and cross-sectional area of mesenteric arteries maintained at 75 mmHg lumen pressure, in calcium-free conditions. *D*: mesenteric artery mRNA expression of the AVP V1A receptor, the endothelin-1 ETA receptor, RGS2, and RGS5 (V1A, RGS2, and RGS5; *n* = 4 male + 5 female control, 4 male + 3 female sRA. ETA, *n* = 4 male control, 4 male sRA). All data are means ± SE. **P* < 0.05 vs. control.

FIG. 7. Serum electrolytes. *A*: serum sodium concentration. *B:* serum-ionized calcium concentration (baseline: *n* = 8 male and 12 female control, 5 male and 8 female sRA; tolvaptan: *n* = 4 male and 5 female control, 4 male and 6 female sRA). All data are means ± SE. **P* < 0.05 vs. control. † *P* < 0.05 vs. baseline sRA.

FIG. 8. Initial ELISA screen of copeptin levels in stored samples of preeclamptic women.

FIG. 9. Maternal plasma copeptin, cystatin C, and vasopressinase (LNPEP) protein concentrations by trimester of pregnancy. (A) Compared to non-pregnant women and women with normotensive pregnancies, plasma copeptin concentrations were significantly elevated in all three trimesters of pregnancy in women that eventually developed preeclampsia. Importantly, copeptin was grossly elevated as early as the sixth week of pregnancy. (B) Plasma cystatin C was affected by gestational age in a similar manner in women that did or did not experience preeclampsia. (C) Plasma LNPEP was essentially unchanged by gestational age and by preeclampsia status. *P<0.05 vs non(pregnant and gestational time(matched control pregnant samples.

FIG. 10. Predictive value of maternal plasma copeptin without adjustment for any covariates. Receiver operator characteristic (ROC) analyses of the utility of copeptin, without correction for covariates, as a predictive tool for the subsequent development of preeclampsia.

FIG. 11. Sufficiency of vasopressin to induce preeclampsia-like phenotypes in C57B1/6J mice. (A) Vasopressin infusion significantly reduced fecundity. X^2 P<0.005. (B) Vasopressin infusion appears to induce hypertension and proteinuria in pregnant mice. (C) Images of example gestational day 18 fetuses, illustrating substantial fetal growth restriction by vasopressin infusion. (D) Electron micrographs of renal cortex, illustrating glomerular endotheliosis. Top two panels are from a saline infused animal which had a glomerular basement membrane thickness within normal limits (thin white arrow). The bottom two panels are from an animal that received vasopressin infusion. Redundant endothelial cell membrane is present (thick black arrow), and basement membranes are markedly thickened with electron dense material (thick white arrow).

DESCRIPTION OF THE INVENTION

[0028] The present invention is based, at least in part, on the discovery that early measurement of copeptin levels during pregnancy is diagnostic of a subject developing preeclampsia later in pregnancy. Furthermore, measurement of serum levels of LNPEP (a leucyl/cystinyl aminopeptidase), which is a zinc-dependent aminopeptidase that breaks down vasopressin and other peptide hormones, is also believed to be diagnostic of a subject developing preeclampsia later in pregnancy. Assays and methods for detection of these indicators of development of preeclampsia may be combined and further coupled with additional assays for preeclampsia including Doppler velocimetry measurements on at least one of a subject's uterine and umbilical arteries. It is further contemplated that additional assays may be combined with those disclosed herein, such as serum screening for aneuploidy, and others known in the art. In this way, a single device may be used to screen for multiple conditions that may affect the mother and/or the fetus.

[0029] In one embodiment of the disclosure, an assay for diagnosing the development of preeclampsia by measuring levels of copeptin and/or LNPEP may further incorporate means for measuring soluble Flt-1 (s-Flt-1) and/or P1GF (placental growth factor), such as antibody-mediated detection and the like. sFlt-1 (soluble fms-like tyrosine kinase-1 - also known as VEGF receptor-1) binds and reduces free circulating levels of the proangiogenic factors VEGF (vascular endothelial growth factor) and P1GF. Additional markers may also be assessed along with or in addition to copeptin to predict preeclampsia including any biomarker associated with preeclampsia, such as, for example, sEng, VEGF, P1GF, uterine artery Dopplers, hCG, inhibin, papp-a, afp, estriol, nuchal translucency, interleukins such as IL-1β or IL-6, high sensitivity C-reactive protein, and PAPP-A.

[0030] In another embodiment of the disclosure, measurement of copeptin levels alone or in concert with other preeclampsia markers in a patient already with the diagnosis of preeclampsia, may be a prognosticator of worsening disease and help a doctor decide further management decisions such as outpatient expectant management, inpatient expectant management, or immediate delivery of the fetus.

[0031] Contemplated kits for diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject may include one or more antibody detection or other assays (test assays) specific for at least the detection of copeptin and/or LNPEP in a sample taken from the subject. The sample is taken early in pregnancy from the subject, for example, in the first trimester of pregnancy. It is further contemplated that later pregnancy stage samples may be measured, as well, including second and third trimesters. In addition, pre-pregnancy samples may also be assessed as controls for pregnant women and/or as predictive samples themselves. While antibody-based detection assays are contemplated herein, additional test assays or detection assays such as copeptin- and LNPEP-specific assays or any that is specific for the protein products of the vasopressin gene are also contemplated herein as are known in the art, including, for example, protein- and/or peptide-specific assays, enzyme activity assays (enzyme detection assays), and mass spectrometry. Kits may further include positive and negative control samples, assay reagents, as well as instructions.

[0032] Samples contemplated in the present disclosure include whole blood, blood fractions, including serum and/or plasma, urine, tissues, cells, and bodily fluids, including, for example, sweat and tears. One preferred sample is plasma. Another preferred sample is serum. Another preferred sample is urine. In one embodiment of the disclosure, a kit includes an antibody detection assay that may be used with plasma, serum or urine, in other words, any bodily sample may be used for the single assay.

[0033] In a further embodiment of the disclosure, an assay may include a test strip, an ELISA, or other antibody-based or other target-specific assay, such as an enzyme activity assay where the presence of a targeted enzyme is detected by chromogenic means and the like due to enzyme activity. Test strips may be prepared in the conventional manner such as is described in U.S. Patent Nos. 6,210,971 or 5,733,787 to Bayer Corporation (Elkhart, IN) . It is contemplated that the test strips may couple attachment of the targeted epitope with the initiation of one or more of a chromogenic, fluorogenic, or luminescent reaction, as is known in the art, to indicate binding of the desired target. Further, a test strip may be characterized as an absorbent substrate capable of immobilizing metabolites bound to a layer of support material. Well-known solid phase supports may include paper, cellulose, fabrics made of synthetic resin, e.g. nylon or unwoven fabric. The absorbent material is typically bound to a layer of support material such as glass fiber or a synthetic polymer sheet to provide structural support. Other suitable solid phase supports are contemplated herein.

[0034] Further, two (or more, such as three or four) assays may be combined in a single assay device, such as, for example a pregnancy test that uses chromogenic or other means (for example, based on urine analysis or other sample). In this embodiment of the disclosure, in addition to the pregnancy test, one or more tests for prediction of preeclampsia would be included. In this embodiment of the disclosure, a "positive" result for pregnancy (the subject is pregnant) may be indicated by a first indicium and a "positive" result for the preeclampsia test (indicating a predisposition for preeclampsia) may be indicated by second indicium.

[0035] In another embodiment of the disclosure, a three test assay is contemplated that tests for pregnancy and multiple preeclampsia predictive markers, such as copeptin and LNPEP. In this way, a greater specificity for prediction of preeclampsia accompanying pregnancy may be had in a single test.

[0036] Test assays may be incorporated into single use devices that may be purchased by the end user (for example, a woman seeking to know whether she is pregnant and at risk for preeclampsia). The test assay devices may be employed by application of a urine, blood, and other some other sample to a single or multiple portions thereof, incubating the test assay for a prescribed period of time, such as about 1 minute, about 5 minutes, about 10 minutes, about 15 minutes, or about 1 hour, and comparing the result to an interpretation key associated with a package in which the test assay device was purchased or on the test assay device itself. A contemplated test device 10 is depicted in FIG. 2. The test device 10 has a substrate 12, a sample application area 14, and a readout area 16. The substrate may, for example, be made of plastic, glass, metal, cellulosic material, a polymer, a cloth, and combinations thereof. The sample application area 14 may be fluidly connected to the readout area 16 within the substrate 12 and/or on a surface of the substrate. Fluid communication may be via one or more microfluidic channels, a wick, a space adapted to cause capillary action, and the like. Upon application of a sample from a subject by a user to the sample application area 14, one or more tests may be engaged or by application of an auxiliary substance (such as for example, a liquid carrier and/or assay reagent). Examples of an auxiliary substance include water, saline, a pH buffering agent, a color changing agent, a protein, an enzyme, and/or a peptide. After an incubation period, the test assay may provide a read out to the user in the read out area 16 that indicates the result of the test(s). Panel B depicts a scenario where the sample applied to the test device 10 came from a pregnant woman (indicated by the plus sign) without predisposition to preeclampsia (indicated by the minus sign). Panel C depicts a scenario where the sample applied to the test device 10 came from a pregnant woman with a predisposition for preeclampsia (double positive signs). Any indicia may be used with the device. Furthermore, a scenario where the sample applied to the test device came from a non-pregnant woman may have double negative signs or no signs at all (not shown).

[0037] Increases in copeptin levels in a sample compared to control are considered to be predictive of the occurrence of preeclampsia during the subject's pregnancy including, for example, of about 1/100 fold, or about 1/50 fold, or about 1/25 fold, or about 1/16 fold, or about 1/8 fold, or about 1/4 fold, or about 2 fold, or greater or less.

[0038] Similarly, decreases in LNPEP levels in a sample compared to control are considered to be predictive of the occurrence of preeclampsia during the subject's pregnancy, including, for example, of about 1/100 fold, or about 1/50 fold, or about 1/25 fold, or about 1/16 fold, or about 1/8 fold, or about 1/4 fold, or about 2 fold, or greater or less.

[0039] In one embodiment of the disclosure, a method of diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject may include collecting a sample, such as, urine, from a subject during the first trimester of pregnancy, measuring copeptin levels in the sample using an antibody detection assay or other assay, and determining whether the subject is likely to develop preeclampsia later in pregnancy by comparing the subject's copeptin levels to a control. Assays may provide data, for example, by color changes, light emission, changes in light emission intensity, densitometry, or changes in opacity/ translucence of a substrate. These data, in turn, may be converted to data points that may be plotted compared to controls.

[0040] The method may further include taking Doppler velocimetry measurements on at least one of the subject's uterine and umbilical arteries.

[0041] By early pregnancy, we mean at least before 20 weeks of amenorrhrea, more preferably, at least before about 16, or about 12, or about 8, or about 4 weeks of pregnancy. Early in pregnancy may also be during the first trimester.

[0042] By "patient" or "subject," it is meant a female subject, such as, a human. Controls contemplated herein may comprise a single healthy pregnant age-matched subject, or a population of multiple healthy pregnant age-matched subject subjects or multiple healthy pregnant subjects, or serum and/or urine samples from a population of multiple healthy pregnant subjects none of whom later develop preeclampsia during pregnancy. In addition, a predetermined control may also be a negative predetermined control. For example, a negative predetermined control comprises one or multiple subjects who developed preeclampsia during pregnancy.

[0043] Antibody detection assays contemplated here may include assays the use antibodies or antibody subparts to target a specific molecule of interest. Detection of the molecule may occur via antibody attachment to the molecule in combination with an indicator associated with the antibody or antibody subpart, such as fluorescent molecule, enzyme, chromagen, chemi-luminescence, or radio chemical, and combinations thereof. It is further envisioned that the molecule of interest, for example copeptin or other AVP gene protein product, may be measured by column chromatography, gas chromatography, mass spectrometry, and combinations thereof.

[0044] One example of an antibody detection assay is an ELISA. An ELISA may include antibodies specific for antigens or epitopes of copeptin or other coexpressed regions of the protein product of the vasopressin (AVP) gene, such as vasopressin and neurophysin II (FIG. 1). An antigen can be a natural or synthetic protein or fragment thereof, polysaccharide, or nucleic acid. Skilled artisans know that antigens can induce an immune response and elicit antibody formation. Antibodies can be molecules synthesized in response to the presence of a foreign substance, wherein each antibody has specific affinity for the foreign material that stimulated its synthesis. The specific affinity of an antibody need not be for the entire molecular antigen, but for a particular site on it called the epitope (Kindt *et al.,* Kuby Immunology, 6th Edition 574 pps, (2006), incorporated herein by reference as if set forth in its entirety). Antibodies can be, for example, a natural or synthetic protein or fragment thereof or nucleic acids (*e.g.*, aptamers) with protein-binding or other antigen-binding characteristics. Antibodies can be produced in response to antigenic stimuli including, but not limited to, exposure to foreign proteins, microorganisms, and toxins. When the panel is contacted with a sample containing at least one antibody specific to an antigen in the panel, an immunocomplex forms between the antigen and the antibody specific for the antigen. One of ordinary skill in the art can assess antigen-antibody immunocomplex formation by techniques commonly used in the art. Examples of suitable additional assays to assess immunocomplex formation contemplated herein include phage immunoblot and radioimmunoassay. *See, e.g.,* (Dubovsky et al., J. Immunother. 30:675-683 (2007).

[0045] Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs and by reference to published texts.

[0046] It should be understood that while various embodiments of the disclosure in the specification are presented using "comprising" language, under various circumstances, a related embodiment of the disclosure may also be described using "consisting of" or "consisting essentially of" language. It is to be noted that the term "a" or "an," refers to one or more, for example, "an immunoglobulin molecule," is understood to represent one or more immunoglobulin molecules. As such, the terms "a" (or "an"), "one or more," and "at least one" is used interchangeably herein.

[0047] The following examples set forth preferred markers and methods in accordance with the disclosure. It is to be understood, however, that these examples are provided by way of illustration and nothing herein should be taken as a limitation upon the overall scope of the disclosure.

EXAMPLES

Example 1. Hypertension in mice with transgenic activation of the brain renin-angiotensin system is vasopressin dependent.

[0048] Activity of the local tissue renin-angiotensin system (RAS) within the brain has been implicated in the development and maintenance of elevated blood pressure in many forms of hypertension. Evidence specifically demonstrating a causal role for brain RAS activity in hypertension comes from various rodent models. These many models include peripheral angiotensin infusion models (59, 77, 100), both elevated (18) and suppressed (36, 48, 65) plasma renin models, psychogenic (55), cold exposure (82), renal injury (96), sleep apnea (16) models, transgenic TGR (mRen2)27 rats (83), and both Dahl salt-sensitive (36) and spontaneously hypertensive rats (SHR) maintained on high-salt diets (47, 61, 95). Two major mechanisms have been documented that account for the blood pressure effects of brain angiotensin. First, actions of the RAS within the supraoptic (SON) and paraventricular hypothalamic nuclei (PVN) stimulate the production and release of arginine vasopressin (AVP, also known as antidiuretic hormone, ADH, or argipressin) (6, 13, 22, 38, 66, 69, 83). Second, hindbrain and brain stem actions of the RAS alter baroreflex function and sympathetic output (28, 32). Interestingly, a population of AVP-expressing neurons project from the PVN to the hindbrain and spinal cord and appear to be involved in the regulation of sympathetic nervous activity, suggesting a possible AVP-mediated cross-talk between these two mechanisms.

[0049] Although some studies have failed to document a substantial role for AVP in blood pressure control in heterogenous groups of human subjects (63), AVP has been implicated as a significant contributor to blood pressure control in selected populations of humans (25, 62). Specifically, African Americans (4), the elderly (19), and patients with congestive heart failure (25) or chronic renal failure (3) all exhibit AVP-dependent hemodynamic changes (9). Importantly, these populations of humans all exhibit low levels of circulating renin (98). As low-renin hypertension accounts for a larger (27%) fraction of human essential hypertensives than high-renin hypertension (16%) (49), it is unclear whether therapeutic targeting of AVP may have been prematurely overlooked as an antihypertensive therapy for selected populations of hypertensive patients.

[0050] Together, these findings have led us to question whether the elevations in AVP are necessary to cause or maintain hypertension due to chronically elevated brain RAS activity and to probe the mechanism(s) of action of AVP in this context. We hypothesized that transgenic activation of the brain RAS would elevate plasma AVP, and that actions of AVP are required to induce hypertension by the brain RAS through some combination of vasoconstriction and altered renal function. To examine these hypotheses, we utilized a unique transgenic animal model previously developed in our laboratory (27, 71). This double-transgenic model (the "sRA" model) takes advantage of the species specificity of the renin-mediated cleavage of angiotensinogen to cause brain-specific hyperactivity of the RAS. We have previously demonstrated that these animals exhibit a robust chronic hypertension, polydipsia, polyuria, and an elevated resting metabolic rate. Importantly, we have also previously determined that sRA mice exhibit elevated plasma AVP levels and a suppression of the circulating RAS despite elevated renal sympathetic nerve activity (27). Here we demonstrate elevated neuronal AVP immunostaining (specifically in the supraoptic nucleus), increased daily secretion of AVP, robust desensitization of the vasculature of sRA mice to AVP, and the necessity of V2 AVP receptor signaling in the maintenance of hypertension and hyponatremia in this model. These findings highlight a major role for AVP in the hypertension of sRA mice.

Materials and Methods:

[0051] *Animals.* All animal work was approved by the University of Iowa Animal Care and Use Committee and was performed in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

[0052] Double-transgenic (sRA) mice were generated as previously described (27, 71). Briefly, "sR" mice expressing human renin under transcriptional control by the neuron-specific synapsin promoter were bred with "A" mice expressing human angiotensinogen under transcriptional control by its own promoter (line 11110/2 X 4284/1). Because of the species specificity of the reaction, human angiotensinogen is only cleaved to form angiotensin I by human renin. Hyperactivity of the RAS is thereby restricted to sites of overlapping transgene expression in sRA offspring (i.e., subsections of the central nervous system that normally produce angiotensinogen).

[0053] *Immunohistochemistry.* Immunohistochemical detection of AVP in the brain was performed on 50 μm thick sections using a rabbit polyclonal antibody to a synthetic peptide corresponding to the first six amino acids of arginine8-vasopressin (Phoenix Pharmaceuticals, Burlingame, CA). Sections were cut from six (3 sRA, 3 wild type) brains perfusion fixed with 4% paraformaldehyde and 0.5% glutaraldehyde and incubated in a 1:1,000 dilution of antibody for 24 h at 4°C. The brains of sRA mice were "notched" for identification and incubated with sections from wild-type animals. After incubation in a biotinylated goat anti-rabbit secondary antibody and avidin-horseradish peroxidase, immunoreactivity was detected using 3,3=-diaminobenzidine as a chromagen. On four sections from each animal, matched for rostrocaudal level, AVP-immunostained fragments larger than 10 μm were counted in the PVN and SON using ImageJ software from the NIH.

[0054] *Blood pressure (tail-cuff).* Here we first examined blood pressure in sRA mice using a Visitech Systems BP-2000 tail-cuff blood pressure monitoring system, as previously described (79). Briefly, animals were acclimated to warmed restraint boxes daily for 1 wk. Once acclimated, 30 measurements of systolic blood pressure were averaged from each animal daily for 2 wk to assess baseline blood pressure. Conivaptan (Vaprisol, YM 087, 22 ng/h sc, Baxter Healthcare) or tolvaptan (OPC-41061, 22 ng/h sc, Sigma Aldrich) was delivered to distinct subsets of mice by osmotic minipump (model 1004, Alzet). After osmotic minipump implantation, pressures were recorded daily for 10 days to assess drug effects.

[0055] *Blood pressure (telemetry).* Radiotelemetric blood pressures were recorded from the carotid artery essentially as previously reported (27). Briefly, a telemeter probe (DSI, model TA11PA-C10) was inserted into the common carotid artery under ketamine-xylazine anesthesia. After >2 days of recovery, blood pressure, heart rate, and spontaneous physical activity were recorded for 30 s every 5 min using the Dataquest program (DSI). After baseline recordings, mice were chronically delivered conivaptan and tolvaptan via osmotic minipump that was implanted through an interscapular incision into the subcutaneous space of the back under isoflurane anesthesia.

[0056] Aortic Vascular Reactivity: Abdominal aortic rings were assessed for vascular reactivity as previously described (30). Briefly, mice were euthanized by overdose of pentobarbital (50 mg, i.p.), and the abdominal aorta was quickly removed and placed in Kreb's buffer containing (in mmol/L): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2 and 11 glucose. Vascular rings (4-5 mm in length) were suspended in oxygenated Kreb's buffer (95% 02 / 5% CO2) in organ baths at 37°C and connected to a force transducer via steel hooks. Resting tension was adjusted to 0.5 grams over 45 minutes. Contractile responses were tested in response to AVP (10^{-10} - 10^{-6} mol/L), phenylephrine (PE, 10^{-8} - $3x10^{-5}$), endothelin-1 (ET-1, 10^{-10} - 10^{-7}), prostaglandin F2α (PGF2α, 10^{-7} - 10^{-4}), and angiotensin II (Ang II, 10^{-10} - 10^{-7}). Following sub-maximal contraction with PGF2α (40-50% of max; $3x10^{-6}$ - $6x10^{-6}$), relaxation responses to acetylcholine (10^{-8} - $3x10^{-5}$) and sodium nitroprusside (10^{-9} - 10^{-5}) were determined.

[0057] Mesenteric Artery Vascular Reactivity: Secondary branches of mesenteric artery were dissected and placed in chilled oxygenated (21% 02, 5% CO2, and 74% N2) Kreb's buffer. A segment (~1 mm long) of artery was transferred to a vessel chamber (DMT), cannulated with glass micropipettes and secured with silk ligatures. The artery was slowly pressurized to 40 mmHg without flow. After 30 min equilibration, vessel viability was tested by constriction response to 100 mM KCl. Vascular responses to PE (10-9 - 10-5 mol/L), AVP (10-12 - 10-7 mol/L), and ET-1 (10-11 - 10-8 mol/L) were then assessed. The artery was then superfused with calcium-free Krebs buffer containing 10-5 mol/L sodium nitroprusside and 2 mmol/L EGTA to maximally dilate the vessel. Internal and external diameters were measured at 75 mm Hg. Wall thickness, media/lumen ratio and cross sectional area (CSA) were calculated as previously described by Neves, et al. (58).

[0058] Gene Expression: Mesenteric arteries (superior mesenteric artery excluded) and kidneys were snap frozen in liquid nitrogen and RNA was extracted in Trizol®. Total RNA was isolated using an RNA Purelink® Minikit (Invitrogen) following the manufacturer's protocol. Concentrations were determined using a NanoDrop ND-1000. cDNA was generated by RT-PCR using SuperScript III® (Invitrogen). qRT-PCR was performed using TaqMan gene expression assays (Applied Biosystems): RGS2 (Mm00501385_m1), RGS5 (Mm00501393_m1), VIA (Mm00444092_m1), ETA (Mm01243722_m1), GAPDH (4352932E), or SYBR188 green assays (primer sequences in Table 1: NKCC2, NCC, NHE3, ENaC-α, FNaC-β, ENaC-γ, NKA-α, V2R, AQP1, AQP2, AQP3, AQP4, PGES, and UT1-A) normalized against β-actin. SYBR-green reagents from Bio-Rad were utilized, and all real time reactions were performed on a Bio-Rad iQ5 iCycle®.

Table 1. SYBR Green primer sequences for quantitative PCR.

Gene	*Primer Sequences*	
NKCC2	*Forward: 5'-CCATGGTAACCTCTATCACTGGGT-3'*	*SEQ ID NO. 1*
	Reverse: 5'-TCAAGCCTATTGACCCACCGAACT-3'	*SEQ ID NO. 2*
NCC	*Forward: 5-AAGTCGGGTGGCACCTATTTCCTT-3'*	*SEQ ID NO. 3*
	Reverse: 5'-TTACGGTTTCTGCAAAGCCCACAG-3'	*SEQ ID NO. 4*
NHE3	*Forward: 5'-TCCTCTCAGCCATTGAGGACATCT-3'*	*SEQ ID NO. 5*
	Reverse: 5'-ACTTTGCTGAGGAACTTCCGGTCA-3'	*SEQ ID NO. 6*
ENaCα	*Forward: 5'-ACAATGGTTTGTCCCTGACACTGC-3'*	*SEQ ID NO. 7*
	Reverse: 5'-TCACGTTGA,4GCCACCATCATCCA-3'	*SEQ ID NO. 8*
ENaCβ	*Forward: 5'-TCTGCCAACCCTGGGACTGAATTT-3'*	*SEQ ID NO. 9*
	Reverse: 5'-TGGCATAGATGCCCTCCTCTCTAA-3'	*SEQ ID NO. 10*
ENaCγ	*Forward: 5'-GCCAATCAGTGTGCAAGCAATCCT-3'*	*SEQ ID NO. 11*
	Reverse: 5'-TTATTTGCTGGCTTTGGTCCCAGG-3'	*SEQ ID NO. 12*
Na-KATPase-α	*Forward: 5'-TGAAGCTGACACCACGGAGAATCA-3'*	*SEQ ID NO. 13*

(continued)

Gene	*Primer Sequences*	
	Reverse: 5'-TGCCGCTTAAGAATAGGCAGGTT-3'	*SEQ ID NO. 14*
V2R	*Forward: 5'-TGTGATTGTCTACGTGCTGTGCTG-3'*	*SEQ ID NO. 15*
	Reverse: 5 '-GGGTTGGTACAGCTGTTAAGGCTA-3'	*SEQ ID NO. 16*
AQP1	*Forward: 5'-CTGGGCATTGAGATCATTGGCACT-3'*	*SEQ ID NO. 17*
	Reverse: 5'-TGATACCGCAGCCAGTGTAGTCAA-3'	*SEQ ID NO. 18*
AQP2	*Forward: 5'-TAGCCCTGCTCTCTCCATTGGTTT-3'*	*SEQ ID NO. 19*
	Reverse: 5'-AAACTTGCCAGTGACAACTGCTGG-3'	*SEQ ID NO. 20*
AQP3	*Forward: 5'-ATGGTGGCTTCCTCACCATCAACT-3'*	*SEQ ID NO. 21*
	Reverse: 5'-AGGAAGCACATTGCGAAGGTCACA-3'	*SEQ ID NO. 22*
AQP4	*Forward: 5'-TGCCAGCTGTGATTCCAAACGAAC-3'*	*SEQ ID NO. 23*
	Reverse: 5'-TCCCATGATAACTGCGGGTCCAAA-3'	*SEQ ID NO. 24*
PGES	*Forward: 5'-TTTGCAACAAGTACTGGCCCATGC-3'*	*SEQ ID NO. 25*
	Reverse: 5'-TGTTCGGTACACGTTGGGAGAGAT-3'	*SEQ ID NO. 26*
UT1-A	*Forward: 5'-CACTGGCGACATGAAGGAATGCAA-3'*	*SEQ ID NO. 27*
	Reverse: 5'-GGGTTGTTGACAAACATCACCTGAGC-3'	*SEQ ID NO. 28*
B-actin	*Forward 5'-CATCCTCTTCCTCCCTGGAGAAGA-3'*	*SEQ ID NO. 29*
	Reverse 5'-ACAGGATTCCATACCCAAGAAGGAAGG-3'	*SEQ ID NO. 30*

[0059] Blood and urine analyses: Plasma was obtained by collecting whole blood by submandibular bleed into lithium heparin coated tubes, then centrifuged at 5,000 x g for 5 minutes, and the supernatant transferred to a fresh tube and frozen at -80°C until analysis. Urine was collected using Nalgene single-mouse metabolism cages as previously described (20). Copeptin was measured using an ELISA kit (USCN Life Sciences), according to the manufacturer's instructions. Blood chemistries and urine creatinine were determined using a handheld iSTAT clinical chemistry analyzer (Abbott), with CHEM8+ cartridges. Urine protein was determined using a bicinchoninic acid assay kit (Thermo Fisher/Pierce), according to the manufacturer's instructions.

[0060] Statistics: Data were analyzed by ANOVA with repeated measures as appropriate. Post-hoc analyses were performed using Bonferroni multiple-comparisons procedures. EC_{50} and maximum response calculations were performed by fitting individual dose-response data sets to a four-parameter logistic function (Hillslope method); y = min + (max - min)/(1+(x/EC_{50})^Hill slope). All mRNA fold changes were calculated using the Livak method (51). All analytical comparisons were performed using SigmaStat / SigmaPlot (Systat). All data are presented as mean ± sem.

Results:

[0061] In both sRA and wild-type animals, AVP immunoreactivity was observed in the cells in the suprachiasmatic (SCN), SON, PVN, and circular nuclei of the hypothalamus as expected (39, 93). AVP-immunoreactive fibers were traceable from the SON and PVN to the median eminence (FIG. 3A). Though there was no obvious difference in the numbers of AVP immunoreactive neurons in the SCN and PVN between sRA and wild-type animals, neuronal and fiber immunoreactivity was consistently denser in the sRA animals. The most striking difference between sRA and control animals was the doubling of the number of AVP immunoreactive neurons detected in the retrochiasmatic part of the SON in sRA animals (FIG. 3A and B) compared with the retrochiasmatic SON in wild-type animals. Copeptin is the COOH-terminal fragment of the fully translated AVP proprotein and is therefore translated in a 1:1 molar ratio with AVP. Because it exhibits a far greater biological half-life than AVP, it has been proposed as a more reliable measure of chronic AVP release than AVP itself (84). Copeptin levels were significantly reduced in plasma from sRA mice (FIG. 3C). Because of its small size (38 amino acids, 4.22 kDa), however, this protein is rapidly cleared from the plasma by the kidneys. Copeptin concentrations appeared elevated in the urine from sRA mice, though the difference was not significantly different. After we accounted for the grossly elevated (~7-fold) urine production rate of sRA mice, however, it is clear that the total daily copeptin clearance into urine is grossly elevated in sRA mice (~20-fold, FIG. 3D). These data together indicate that there is an approximate 20-fold increase in AVP secretion in sRA mice. This large difference in total daily copeptin loss to urine was still present (8-fold) after normalization for total daily urine creatinine (creatinine: control, 0.20 ± 0.03 vs. sRA, 0.40 ± 0.05 mg/day, $P < 0.01$, and copeptin/creatinine: control, 49 ± 36 vs. sRA, 406 ± 176 pg/mg, $P = 0.05$) or (10-fold) after normalization total daily urine protein (protein: control, 41 ± 4 vs. sRA, 86 ± 9 mg/day, $P < 0.01$, and copeptin/protein: control, 180 ± 100 vs. sRA, 1,845 ± 665 mg/mg, $P = 0.02$).

[0062] Under baseline conditions, sRA mice exhibited a hypertension that was easily detectable by tail-cuff (FIG. 4A). These data replicate our previously published measures of hypertension in this model, as determined by direct cannulas and by radiotelemetry (27, 71). Chronic subcutaneous infusion of the nonselective, nonpeptide AVP V1A/V2 receptor antagonist conivaptan resulted in a complete normalization of the hypertension in sRA mice. Continuous recording of blood pressures in an exemplar sRA mouse at baseline and during 18 days of continuous subcutaneous conivaptan infusion documented a gradual but substantial reduction in blood pressure (FIG. 4*B*) that was paralleled by a slight reduction in heart rate (FIG. 4*C*). Importantly, spontaneous physical activity remained normal throughout the recording period, suggesting that the animal was not lethargic or otherwise ill due to the surgery and conivaptan infusion (FIG. 4D).

[0063] To dissect the relative contributions of various vasopressin receptor subtypes in the hypertension of sRA mice, we next examined the blood pressure consequences of chronic subcutaneous infusion of the V2-selective antagonist tolvaptan. Chronic infusion of tolvaptan caused a nearly identical normalization of blood pressure (FIG. 4*E*) to that observed with conivaptan (FIG. 4*A*). Importantly, this blood pressure reduction was confirmed in a cohort of sRA mice tested using radiotelemetry (FIG. 4*F*).

[0064] Additional evidence for chronic hypertension and vasopressin-specific changes in sRA mice comes from vascular reactivity assays. First, abdominal aortic rings were examined ex vivo for reactivity to selected vasoconstrictor and vasodilator compounds. Aortic rings from sRA mice exhibited normal constrictor responses to potassium chloride (FIG. 5*A*). Abdominal aortic rings exhibited a robust rightward shift in responses to the vasodilator acetylcholine (Table 2), but normal responses to sodium nitroprusside, indicating endothelial dysfunction typical in chronic hypertension models (FIG. 5, *B* and *C*). Supporting a chronic elevation in AVP levels, abdominal aortas from sRA mice exhibited a robust suppression of constrictor responses to AVP (FIG. 5*D*), reflected both in a trend toward a rightward (reduced) potency shift and a significant suppression of maximal response (Table 2). No potency or efficacy changes were observed in contractile responses to PE, ET-1, ANG II, or PGF$_{2\alpha}$ (FIG. 5, *E-H*), suggesting AVP specific changes in the sRA vasculature.

[0065] Table 2. Potency and efficacy analyses of various vasoactive compounds in abdominal aortas and 2°-branch mesenteric artery of male sRA and control littermate mice.

[0066]

Table 2. ***Potency and efficacy analyses of various vasoactive compounds in abdominal aortas and 2°-branch mesenteric artery of male sRA and control littermate mice***

	EC$_{50}$		Maximum Response	
Compound	**Control**	**sRA**	**Control**	**RA**
Abdominal aorta	*nmol/l*	*nmol/l*	*g*	*g*
Phenylephrine	1,900 ± 250	2.430 ± 620	1.18 ± 0.05	1.02 = 0.10
Angiotensin II	2.26 ± 0.45	4.24 ± 1.14	0.32 ± 0.03	0.38 ± 0.03
Arginine vasopressin	2.19 ± 0.19	3.72 ± 0.73*	0.29 ± 0.03	0.12 ± 0.01
Prostaglandin F$_{2a}$	4.600 ± 750	3.600 ± 290	1.59 = 0.06	1.50 ± 0.10
Endothelin-1	3.40 ± 0.33	5.19 ± 0.93	0.45 ± 0.04	0.55 ± 0.09
	nmol/l	*nmol/l*	%	%
Acetyleholine	130 ± 35	879 ± 298*	78.9 ± 3.3	70.4 ± 8.9
Sodium nitroprusside	29.4 ± 3.7	34.2 ± 1.7	91.5 ± 0.5	93.5 ± 0.8
Mesenteric artery	*nmol/l*	*nmol/l*	%	%
Phenylephrine	1.460 ± 660	560 ± 80	67.3 = 3.0	64.5 ± 4.1
Arginine vasopressin	0.57 ± 0.15	15.89 ± 13.46	52.5 = 3.2	17.3 ± 6.5†
Endothelin-t	1.68 ± 0.43	0.45 ± 0.17*	67.8 ± 4.3	58.0 ± 4.6

Data are presented as means ± SE. Aortas: Control. *n* = 6: sRA. *n* = 5. Mesenteric artery : Control. *n* = 6; sRA. *n* = 6. *$P \leq 0.05$. and †$P \leq 0.001$ vs. Control

[0067] Acknowledging that smaller arteries are important in controlling peripheral resistance, vascular reactivity of second order branches of mesenteric arteries were next examined using pressurized myography. Mesenteric artery branches exhibited a significant reduction in contractile response to potassium chloride (FIG. 6*A*); however, normalization of other constrictor responses to this lower KCl response in sRA mice had no qualitative effect on data interpretation (not shown). Similar to abdominal aortic rings, mesenteric arteries from sRA mice exhibited a trend toward a rightward shift and a substantial suppression of maximal response (Table 2) to AVP (FIG. 6*B*).

[0068] Mesenteric arteries exhibited normal contractile responses to PE, with no change in efficacy or potency. In response to ET-1, mesenteric arteries from sRA mice exhibited a normal maximal response and a small but statistically significant leftward potency shift. These data confirm an AVP-specific desensitization in smaller arteries of sRA mice, further supporting the conclusion that AVP is chronically elevated in sRA mice. Mesenteric arteries from sRA mice exhibited substantial eutrophic inward remodeling, providing further evidence of chronic hypertension in this model. While no difference in external diameter was detected between control and sRA mice (FIG. 6C), lumen diameter was significantly smaller in sRA mice because of increased wall thickness. This resulted in an increased media-tolumen ratio but no significant change in cross-sectional area.

[0069] To explain the reduced vascular reactivity to AVP, we next measured expression of the V1A receptor. Mesenteric arteries from sRA mice exhibited significantly suppressed V1A receptor mRNA but no change in ETA receptor expression (FIG. 6D). Furthermore, there was a selective downregulation of regulator of G protein signaling-2 (RGS2) expression but no change in RGS5 expression.

[0070] In contrast to vascular V1A downregulation, renal V2 receptors and aquaporin-2 mRNA levels were unchanged in sRA mice (Table 3). The only renal transporter that showed significant changes in expression in sRA mice was the sodium chloride cotransporter (NCC, 5-fold of control, $P < 0.05$), though the sodium/hydrogen exchanger (NHE) showed a trend toward reduction (NHE3, 0.6-fold of control, $P = 0.08$) and the ENaC-α subunit showed a trend toward elevation (ENaC-α, 10-fold of control, $P = 0.08$). It should be noted that these renal gene expression assays were performed on only male sRA and littermate control mice, and the statistical power is low due to a small number of replicates per group ($n = 4$ each). Thus it is possible that the changes in NHE3 and ENaC-α may both be physiologically significant.

[0071] Table 3. Renal expression of selected receptors and transporters in sRA and littermate control mice.

Table 3. *Renal expression of selected receptors and transporters in sRA and littermate control mice*

Gene	Control (*n* = 4=	sRA (*n* = 4)	*i*-Test *P* Value
AVPR2	1.000 (0.840-1.191)	0.778 (0.583-1.038)	0.425
NCC	1.000 (0.736-1.359)	5.232 (3.850-7.112)	0.009
NHE3	1.000 (0.825-1.212)	0.605 (0.522-0.701)	0.075
NKCC2	1.000 (0.632-1.581)	0.621 (0.415-0.929)	0.464
ENaCα	1.000 (0.468-2.135)	10.021 (4.556-22.041)	0.080
ENaCβ	1.000 (0.657-1.522)	0.596 (0.282-1.263)	0.611
ENaCγ	1.000 (0.669-1.495)	1.682 (1.120-2.525)	0.398
Na-K-ATPase-α	1.000 (0.620-1.613)	0.819 (0.553-1.215)	0.744
AQP1	1.000 (0.610-1-640)	0.643 (0.511-0.808)	0.441
AQP2	1.000 (0.747-1.338)	0.541 (0.440-0.665)	0.133
AQP3	1.000 (0.636-1.571)	0.317 (0.192-0.524)	0.640
AQP4	1.000 (0.397-2.521)	0.483 (0.237-0.983)	0.506
PGES	1.000 (0.779-1.283)	0.616 (0.237-1.597)	0.164
UT1-A	1.000 (0.486-2.057)	0.569 (0.406-0.799)	0.643

Data are presented as fold-of-control: means ± SE. See text for abbreviations and more information.

[0072] Finally, to more directly probe a V2-mediated mechanism in the cardiovascular phenotypes of sRA mice, we examined blood chemistry responses to tolvaptan (Table 4). We previously documented an approximate 4 mM hyponatremia in sRA mice under baseline conditions (27). Here we determined that sRA mice were hyponatremic (FIG. 7*A*) and hypocalcemic (FIG. 7*B*), and chronic tolvaptan delivery corrected both of these imbalances (genotype X drug interaction $P < 0.05$ for both). sRA mice also exhibited alterations in chloride, total CO2, glucose, blood urea nitrogen, creatinine, hematocrit, and anion gap, and whereas tolvaptan treatment did affect some of these endpoints (potassium, chloride, and blood urea nitrogen), it did so in a manner independent of genotype as no genotype X drug interactions were uncovered (Table 4).

[0073] Table 4. Blood chemistry at baseline or following tolvaptan infusion in sRA and control littermate mice.

[0074]

Table 4. *Blood chemistry at baseline or following tolvaptan infusion in* ***sRA*** *and control littermate mice*

	Females				Males			
	Baseline		Tolvaptan		Baseline		Tolvaptan	
Parameter	Control (*n* = 12)	sRA (*n* = 8)	Control (*n* ± 5)	sRA (*n* = 6)	Control (*n* = *8*)	sRA (*n* = 5)	Control (*n* = *4*)	sRA (*n* = *4*)
Females								
Age, wk	23.5 ± 0.1	23.6 ± 0.2	22.7 ± 0.2	22.6 ± 0.2	19.4 ± 1.1	18.5 ± 1.3	22.5 ± 0.3	22.5 ± 0.3
Sodium, mM$^{G.T. G.T}$	147.3 ± 0.7	142.6 ± 1.2	146.2 ± 0.7	146.3 ± 0.7	147.4 ± 0.9	141.4 ± 2.8	148.3 ± 1.3	147.5 ± 1.9
Potassium, mM^{T}	6.6 ± 0.3	6.5 ± 0.5	6.4 ± 0.1	5.3 ± 0.3	6.5 ± 0.5	6.8 ± 0.7	6.6 ± 0.5	5.4 ± 0.1
Chloride. mM$^{G.S.T}$	115.7 ± 1.0	110.3 ± 1.7	112.4 ± 0.5	105.2 ± 1.6	119.4 ± 2.0	115.8 ± 2.2	115.5 ± 0.3	107.0 ± 2.7
Ionized calcium. mM$^{G.T.G.T}$	1.15 ± 0.04	1.01 ± 0.04	1.19 ± 0.05	1.18 ± 0.03	1.07 ± 0.06	0.78 ± 0.12	1.18 ± 0.04	1.18 ± 0.03
Total CO_2, mM$^{G. T}$	18.4 ± 1.1	21.6 ± 2.1	24.0 ± 1.1	28.8 ± 1.7	17.0 ± 1.2	18.8 ± 1.7	24.0 ± 0.8	26.0 ± 3.4
Glucose. mg/dl$^{G. S-T}$	215 ± 8	193 ± 16	190 ± 13	136 ± 15	196 ± 11	155 ± 16	182 ± 7	181 ± 28
BUN, mg/dl$^{G.T}$	22.4 ± 1.5	42.4 ± 4.9	18.4 ± 0.9	24.5 ± 1.7	28.5 ± 4.0	40.8 ± 9.0	21.5 ± 0.9	27.3 ± 2.1
Creatinine, mg/dl^{G*}	0.21 ± 0.01	0.33 ± 006	0.24 ± 0.02	0.33 ± 0.04	0.21 ± 0.01	0.24 ± 0.02	0.25 ± 0.03	0.30 ± 0.04
Hematocril. ^{c}*k* *R*BC$^{G. G.S}$	45.8 ± 0.5	52.6 ± 0.6	45.4 ± 0.7	49.8 ± 1.1	43.8 ± 0.7	52.6 ± 0.9	43.8 ± 1.1	52.0 ± 1.1
Anion gap. mM$^{G. G\times S}$	21.2 ± 0.6	18.4 ± 1.0	17.0 ± 1.5	18.5 ± 0.7	18.3 ± 1.4	14.8 ± 4.4	16.3 ± 0.5	21.0 ± 2.4

Values are means ± SE. Three-way ANOVA results: $^{G}P < 0.05$ main effect of genotype. $^{5}P < 0.05$ main effect of sex. $^{T}P < 0.05$ main effect of tolvaptan (22 ng/h. 10 days sc). $^{G\times 5}P < 0.05$ genotype × sex interaction. $^{G\times T}P < 0.05$ genotype × tolvaptan interaction. $^{S\times T}P < 005$ sex × tolvaptan interaction. *Lower detection limit for creatinine assay was 0.20 mg/dl; values below detection were assigned value of 0.20. All end points were evaluated from check capillary blood collected in lithium-heparin coated tubes and tested using CHEM8+ cartridges in an iSTAT handheld chemistry analyzer (Abbott Labs).

Discussion

[0075] Here we examined a unique double-transgenic mouse model to test the hypothesis that AVP is required for the hypertension induced by the brain RAS. Immunohistochemical examination of the brain revealed elevated AVP levels in the retrochiasmatic part of the supraoptic hypothalamic nucleus but no consistent change in PVN immunostaining in sRA mice. Confirming a required role for AVP signaling in the hypertension, chronic blockade of vasopressin V1A/V2 receptors resulted in normalization of blood pressure in sRA mice. While vascular reactivity in multiple arteries to PE, ET-1, ANG II, and $PGF_{2\alpha}$ were largely unchanged in sRA mice, responses to AVP were greatly desensitized. Selective inhibition of V2 receptors had a potent antihypertensive action in sRA mice and normalized the hyponatremia typical of this model. Together, these data strongly support a required role for AVP, acting at V2 receptors, in the maintenance of brain RAS-derived hypertension.

[0076] Increased AVP signaling has been suggested as a mechanism for the hypertension in many models. Mice with either tightly regulated or strongly overexpressed transgenic hyperactivity of the RAS throughout the body require elevated AVP signaling to maintain hypertension (17, 57). Deoxycorticosterone acetate (DOCA)-salt hypertension, which is dependent on elevated brain RAS activity (38, 48, 65), also depends on AVP signaling. DOCA-salt treatment results in elevated plasma AVP levels (14, 54, 56, 92). Intracerebroventricular infusion of the angiotensin-converting enzyme inhibitor captopril into rats both prevented and reversed DOCA-salt hypertension and was associated with a reduction in plasma vasopressin levels despite a reduced blood pressure (38). The dependence of DOCA-salt hypertension on

AVP has also been demonstrated using AVP-deficient Brattleboro rats, as the hypertensive effects of DOCA-salt are greatly diminished in these animals (14, 99). Complimenting these findings from various hypertensive models, TGR(AS-rAOGEN) rats, which exhibit reduced glial production of angiotensinogen, are hypotensive and have reduced plasma AVP levels (74). These animals also exhibit altered patterns of AVP V1A receptor expression within the brain (11), further supporting a brain RAS-AVP interaction. Mice deficient for the V1A AVP receptor are hypotensive, though the relative importance of brain, vascular, cardiac, thrombocyte, and hepatic receptors is unclear (2, 46).

[0077] Effects of the RAS on the production and release of AVP were reported as early as 1970, when Bonjour and Melvin (6) demonstrated that peripherally administered renin or angiotensin II resulted in dose-dependent increases in plasma AVP in dogs. Evidence for direct actions of angiotensin on AVP release within the brain was provided by ex vivo experiments using isolated rat neurohypophysis (22). Electrolytic lesion of the subfornical organ (37) or transection of efferent projections from the subfornical organ (45) both attenuate the release of AVP into the plasma in response to intravenous ANG II. Thus the demonstrations here of elevated brain AVP staining and increased daily copeptin (and thereby AVP) release in sRA transgenic mice were expected. Further work is required to causally link specific RAS receptor subtypes to the AVP elevation, as morphological and functional evidence support roles for both AT1 and AT2 receptors in AVP release.

[0078] The strongly increased AVP immunoreactivity in the SON implicates ANG II-mediated hyperactivity in the supraopticneurohypophysial pathway as leading to elevated AVP in sRA mice. ANG II injections into the SON depolarize neurosecretory cells (93), ANG II-immunoreactive neurons and axon terminals are found in the rodent SON intermingled with AVP immunoreactive neurons, and ANG II and AVP are colocalized in some neurons (39). It is thus likely that local production and/or actions of ANG II within the SON regulate AVP production and secretion.

[0079] AVP is an endogenous agonist for at least four subtypes of receptors. The V1A receptor subtype is primarily found in the vasculature, signals primarily through Gaq, and mediates vasoconstriction. V1A receptors are also present in neurons and appear to signal through cAMP to regulate neuronal function (2, 89). The V1B receptor subtype is primarily found in the brain, signals through Gaq, and stimulates adrenocorticotropic hormone. The V2 receptor subtype is primarily found in the collecting duct of kidney nephrons, signals through Gas, and stimulates water reabsorption through aquaporin mobilization. There is some evidence for expression of V2 receptors in extrarenal tissues such as lung (20) and cerebellum (43), though their physiological significance in these tissues is unclear. Finally, AVP is also an agonist at the VACM-1 receptor, also known as Cullin-5, where it elicits calcium mobilization in endothelial cells and renal collecting ducts (7, 8). Our determination that mesenteric artery V1A receptors were downregulated in sRA mice but renal V2 receptor expression was unchanged may suggest a greater role for AVP-mediated renal water retention in the hypertension of sRA mice. Though not directly tested herein, this conclusion is supported by the slow time course for the effects of conivaptan (several days of infusion to see an effect, FIG. 3B), the antihypertensive effects of tolvaptan (FIG. 3, E and F), and the normalization of baseline hyponatremia and hypocalcemia in this model (Table 4 and FIG. 6) that are typical of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) (24). RGS2 is expressed throughout the cardiovascular system and acts to negatively regulate Gaq-mediated GPCR signaling, and therefore oppose vasoconstrictor responses (75). Studies in human patients have revealed a negative correlation between RGS2 expression and blood pressure, with hypertensive patients showing reduced RGS2 expression and hypotensive patients exhibiting elevated RGS2 expression (35, 75, 94). A similar correlation is observed in hypertensive animal models (10, 11) and was again observed in the present study (FIG. 5D). RGS2 is known to be regulated in a tissue-specific manner, and within cardiovascular tissues RGS2 is controlled through multiple biphasic mechanisms (97). Acute activation of Gaq by various hormone/receptor combinations upregulates RGS2 rapidly, possibly to serve as a negative feedback mechanism. In contrast, chronic stimulation of Gaq systems appears to cause a tonic suppression of RGS2 expression (10, 11, 88, 97). Mice deficient for RGS2 exhibit robust hypertension due to chronic increases in peripheral vasoconstriction (31, 34). Vasopressin-induced calcium transients in vascular smooth muscle cells from RGS2 knockout mice are augmented, highlighting the relationship between RGS2 and AVP signaling, presumably through V1A receptors (86) as these receptors utilize Gaq signaling (2, 89). RGS2 knockout mice also exhibit substantially greater end-organ damage from chronic hypertension than do wild-type animals (81). RGS2 also attenuates cAMP signaling in the kidney through modulation of adenylyl cyclases (29, 85), which may result in modulation of AVP signaling through V2 receptors, Gas, and cAMP. Indeed, modulation of RGS2 greatly affects renal V2 receptor signaling and the renal effects of AVP in vivo (72). Thus it is tempting to speculate that modulation of RGS2 in various tissues, along with elevated AVP signaling, may contribute to the maintenance of hypertension in the context of chronically elevated brain RAS activity. Differential regulation patterns for V1A receptors and V2 receptors in pathological states have previously been described. Gózdz et al. (26) previously demonstrated that in the TGR(mRen2)27 rat model of high-renin hypertension, cardiac V1A receptors are upregulated compared with control Sprague-Dawley rats, while renal V2 receptors are unchanged between strains. Trinder et al. (87) previously demonstrated that in the streptozotocin-injection model of Type 1 diabetes mellitus, rats exhibited reduced hepatic and renal expression of V1 receptors and AVP-induced inositol phosphate production, while renal V2 receptors and AVP-induced cAMP production are again unchanged. Thus our observation that vascular V1A receptors were downregulated and vascular reactivity to AVP was desensitized while renal V2 receptors and their function were largely unchanged is not unprecedented.

[0080] Previously, we demonstrated a robust (twofold) elevation in plasma AVP levels in female sRA mice under baseline conditions (collected 4 h into the light phase of a 12:12 light-dark cycle), and this difference was not detected in males (27). The doubling of plasma AVP concentration was achieved in sRA males as well, following a very brief (4 h) water restriction that had no effect on plasma AVP in control males. In the present study we determined that copeptin loss to urine (the major mechanism for clearance of this 4-kDa peptide) was the same in both male and female mice (FIG. 2D). While urine copeptin measures relate to the rate of AVP secretion, direct plasma AVP measures relate to both AVP secretion and degradation/clearance. Thus we now hypothesize that AVP secretion rates are similarly elevated in both male and female sRA mice, but that there exist sex-specific differences in the rates of AVP degradation/clearance. The determination that AVP receptor blockade effectively eliminated hypertension in both sexes in the present study (FIG. 3) further supports this hypothesis. Studies into the sex-specific differences in AVP clearance mechanisms are ongoing.

Perspectives and Significance

[0081] Collectively, our data support a model of elevated brain RAS activity driving an increase in AVP secretion. AVP action upon V2 receptors subsequently contributes to elevated blood pressure and hyponatremia. We hypothesize that these effects are mediated through excessive water retention, which when combined with the extreme polydipsia of this model, results in a polyuria phenotype possibly through a pressure-diuresis mechanism. Based on the well-known function of V2 receptors in renal collecting duct aquaporin-2 mobilization, we suspect a renal-mediated mechanism is hyperactive in sRA mice, though we have not here directly examined the localization of the V2 receptors responsible for the observed antihypertensive actions of tolvaptan. These data may support the use of the sRA mouse as an experimental model of the SIADH (24) or other diseases characterized by elevated AVP production or reduced clearance. The brain-specific generation and action of angiotensin peptides is gaining substantial interest for the regulation of cardiovascular function, fluid balance, metabolic control, and even learning and memory. Vasopressin is also well-recognized for its role in fluid balance, blood pressure regulation, and various behaviors (pair bonding, altruism, learning, memory, fluid, and food intake), and its production and release are well known to be stimulated by angiotensins within the brain. Therefore, the implication of vasopressin as a primary mediator of angiotensinergic hypertension simultaneously 1) identifies vasopressin as a possible mediator of other newly recognized functions of the brain RAS (e.g., metabolic control, learning and memory, etc.); and 2) identifies angiotensinsensitive, vasopressin-producing brain structures (e.g., the supraoptic nucleus) as major cardiovascular regulatory centers that may deserve substantially more investigation for therapeutically targeting hypertension and other disorders, especially in selected human populations with low-renin hypertension (3, 4, 9, 19, 25, 49, 62, 63, 98).

Example 2. Copeptin as an early clinical screen for mothers likely to develop preeclampsia.

[0082] Pregnant women were recruited from the beginning of their prenatal care. Women who enrolled into the Maternal Fetal Tissue Bank (MFTB) provided a maternal blood sample for research whenever they had clinically indicated blood draws throughout pregnancy. In addition, amniotic fluid samples, urine samples, fetal cord blood, and placental tissue from clinically indicated tests or procedures are collected and are utilized for research. Short and long term clinical information regarding the health of the mother and child were also extracted to correlate with the samples. To date, over 1500 women are currently enrolled in the MFTB with over 25,000 aliquoted samples.

[0083] An initial ELISA screen of copeptin levels in stored samples of preeclamptic women demonstrated the trend of increasing copeptin levels by the third trimester (FIG. 8).

Example 3. Early First Trimester Prediction of Preeclampsia by Copeptin: Is Vasopressin Hypersecretion an Initiating Event in the Pathogenesis of Preeclampsia?

[0084] Preeclampsia affects 5-7% of all pregnancies, approximately 300,000 per year in the U.S. Yet, it disproportionately causes 15% of all maternal-fetal morbidity and mortality (73). Preeclampsia is known to cause immediate and long term maternal-fetal morbidities such as fetal growth restriction, maternal-fetal death, and future adult neurological and cardiovascular diseases for mother and child (23, 40, 52, 53, 90, 91). Because its pathogenesis is poorly understood, preventative, therapeutic, and curative modalities for preeclampsia are elusive. This emphasizes the importance of finding appropriate unifying pathways to be able to predict and treat preeclampsia. One potential pathway is the vasopressin pathway.

[0085] Vasopressin exhibits a short biological half-life (on the order of 5-20 minutes in blood), which complicates direct measurement of this hormone. Vasopressin is translated in 1:1 stoichiometric ratio with a small, inactive pro-segment, copeptin. Copeptin is eliminated primarily by renal excretion and is very stable in plasma. Consequently, it is a very useful biomarker for vasopressin secretion (3). Zulfikaroglu et al. (101) recently documented a late second/early third trimester elevation in circulating copeptin in preeclamptics. Furthermore, selected populations exhibit vasopressin-dependent hypertension, including African Americans, the elderly, and patients in chronic heart or renal failure (3, 4, 9, 19). These populations are also characterized by low circulating renin-angiotensin system activity. Interestingly, relative to normotensive pregnancies, preeclamptic pregnancies also exhibit reduced circulating activity of the renin-angiotensin system (33). These data lead us to hypothesize a potential causative role for vasopressin hypersecretion in the development of preeclampsia, and the possible utility of copeptin as a novel predictive biomarker for preeclampsia in early pregnancy.

METHODS

[0086] Biosample and Clinical Data Acquisition: Maternal plasma and clinical patient information were obtained through the University of Iowa IRB-approved (IRB# 200910784) Maternal Fetal Tissue Bank (MFTB). In this bank, pregnant women are prospectively recruited from the beginning of their prenatal care. MFTB inclusion criteria include any women > 18 years old receiving prenatal care at the University of Iowa Hospitals & Clinics who are English speaking. The MFTB exclusion criteria include prisoners, HIV+ or Hepatitis C positive women. Women who enroll into the MFTB provide a maternal blood sample for research whenever they have clinically indicated blood draws throughout pregnancy. All maternal blood in the MFTB is uniformly processed. Maternal plasma and the buffy coat are isolated, aliquoted, and stored at -80°C. Maternal and neonatal clinical data obtained by the MFTB is obtained via data extraction from the electronic medical record using standardized data extraction forms. Extracted clinical data is routinely monitored for accuracy and completeness by two of the authors (MKS and DAS). Additional data is also extracted by bioinformatics collaborators from the University of Iowa Institute for Clinical and Translational Science who are able to query the central electronic medical record database.

[0087] Cohort Assembly: Inclusion criteria for preeclampsia cases included women who delivered at UIHC, were enrolled in the MFTB, and carried the diagnosis of preeclampsia at the time of delivery. The diagnosis and classification of cases of preeclampsia were based on the standard American College of Obstetrics and Gynecology (ACOG) definitions for analysis (1). These cases were identified by cross-referencing the MFTB database with the bioinformatics query of mild and severe preeclampsia ICD-9 codes (642.4x, 642.5x, 642.7x, 642.9x) of bank participants at the time of delivery. The electronic medical record of each potential case was evaluated to confirm the diagnosis of preeclampsia by the ACOG definitions. Maternal age-matched plasma samples and corresponding clinical data for the control population were obtained by utilizing the MFTB database. The gestational age at the time of the collection of the samples were classified by trimesters: first trimester (< 13 completed gestational weeks), second trimester (13-26 completed gestational weeks), and third trimester (>26 weeks).

[0088] Procedures: All maternal plasma copeptin concentrations were measured in duplicate using a commercial enzyme-linked immunosorbent assay (ELISA) specific for human copeptin (USCN Life Science, Inc, Houston, TX). The assay was performed according to the manufacturer's instructions. The minimum detectible dose of human copeptin for this assay was 5.4 pg/mL. The intra-assay coefficient of variation is < 10% and the interassay coefficient of variation is < 12%. To examine if renal function or vasopressin degradation throughout pregnancy affected copeptin concentration, plasma Cystatin C (Sigma-Aldrich, St. Louis, MO) and vasopressinase (LNPEP, USCN Life Science, Inc, Houston, TX) were measured in duplicate in all samples utilizing commercial ELISA kits.

[0089] Animal Studies: Female C57B1/6J mice were obtained from Jackson Laboratories between 8-12 weeks of age. Osmotic minipumps infusing vasopressin (240 ng/hr) or saline vehicle were inserted into the subcutaneous space via incision between the scapulae. Following three days of recovery, females were mated with male C57B1/6J mice. Presence of a vaginal plug indicated gestational day 0.5. Blood pressure was tracked before mating and throughout gestation by tail-cuff plethysmography. On gestational day 18, females were sacrificed for necropsy. Pup weight was recorded. Dam kidney sections were generated and imaged by electron microscopy by the University of Iowa Department of Pathology. All studies were approved by the University of Iowa Animal Care and Use Committee (ACURF# 1211239).

[0090] Statistical Analyses: The major aim of this study was to determine if differences in first-trimester copeptin concentrations between pregnant women who did and did not develop preeclampsia predicted the development of preeclampsia. Using the smallest effect size in late gestation maternal plasma copeptin concentrations from Zulfikaroglu *et al.* (101) between control (310 pg/mL) and mild preeclamptics (620 pg/mL) with the largest reported standard deviation of 180 pg/mL, power of 80% and $\alpha = 0.05$, only 7 participants per group are required. In order to account for a parsimonious, mixed effects regression model of 3 variables, a minimum of 30 samples per group was utilized.

[0091] All statistical analyses were performed with SigmaPlot 12.0 software (Systat Software, Inc, California) and confirmed using SAS 9.1 software (SAS Institute Inc, Cary, NC). Stepwise regression was used to develop a model for this dataset and to evaluate for possible confounding. Logistic regression models were constructed and receiver operating characteristic curves were constructed for regression diagnostics. In addition, chi square or Fisher exact test was utilized for categorical variables. For continuous variables, the Student's t-test or if criteria for normality were not met, Mann-

Whitney test was utilized. All variables were tested at significance level of 0.05.

RESULTS

[0092] A total of 30 individual control (C) subjects and 51 individual preeclamptic (P) subjects were utilized in this study. A full complement of first (C=12, P=20), second (C=10, P=20), and third (C=30, P=51), trimester plasma samples were not available for each participant. Maternal age, gravida, body mass index, percentage of those with chronic hypertension and preexisting diabetes were similar between the control and preeclamptic groups (Table 5). In addition, the racial distribution between these groups were also similar and reflective of the Iowa population with a predominantly Caucasian populace based on current Iowa census data. Of these maternal characteristics, only history of preeclampsia was significantly higher in the control group vs. the preeclamptic group (53.3% vs. 17.7%, p=0.002). When evaluating the delivery characteristics between the two groups (Table 5), typical differences were observed between groups. The preeclampsia group exhibited a significantly lower gestational age at delivery (36.2 vs. 38.7 weeks, P=0.001), higher percentage of twin gestation (21.6 vs. 0%, P=0.016), and lower birthweight (2777.0 vs. 3424.0 grams, P=0.0001). These findings are consistent with the known morbidities associated with preeclampsia: higher rate of preterm delivery, higher rate of twin gestation, and lower birthweight due to vascular causes and earlier delivery (80).

Table 5. Group Characteristics.

Group Characteristics	Nonpregnant (n=33)	Control (n=31)	Preeclampsia (n=50)	P Value
Maternal Characteristics				
Maternal Age (years)	31.4	30.0	30.0	0.86
Gravida	1.3	2.6	2.7	<0.001
Body Mass Index (kg/m^2)	29.6	30.4	31.9	0.48
Chronic Essential Hypertension	9.1%	29.0%	20.0%	0.13 (χ^2=4.1)
Preexisting Diabetes	3.0%	9.7%	10.0%	0.47 (χ^2=1.5)
History of Preeclampsia	0%	51.6%	18.0%	0.002 (χ^2=25.7)
Race: Caucasian, not Hispanic	90.9%	87.1%	90.0%	0.63 (χ^2=6.2)
Race: Hispanic	0%	6.5%	4.0%	0.63 (χ^2=6.2)
Race: Asian	6.1%	3.2%	0%	0.63 (χ^2=6.2)
Race: African-American	3.0%	3.2%	2.1%	0.63 (χ^2=6.2)
Pregnancy Characteristics				
Gestational Age at Delivery (wk)		38.7	36.2	0.001
Mode of Delivery: Vaginal		53.3%	34.0%	0.09 (χ^2=4.75)
Mode of Delivery: C-Section		40.0%	64.0%	0.09 (χ^2=4.75)
Mode of Delivery: Operative Vaginal Delivery		6.7%	2.0%	0.09 (χ^2=4.75)
Twin Gestation		0%	21.6%	0.016 (χ^2=5.76)
Birthweight (grams)		3424.0	2777.0	< 0.001
1 minute APGAR		7.2	7.3	0.95
5 minute APGAR		8.7	8.5	0.49

[0093] As seen in FIG. 9A, measurement of the maternal plasma copeptin concentration revealed a significant increase in mean copeptin in pregnant women who developed preeclampsia in comparison with control, non-preeclamptic women in the first trimester (2045 vs. 903 pg/mL, p=0.008), second trimester (1806 vs. 706 pg/mL, p=0.001), and third trimester (1890 vs. 822 pg/mL, p=0.0006). These group differences in plasma copeptin are likely not associated with changes in renal function and vasopressin degradation as measured by plasma Cystatin C and Vasopressinase respectively as these levels were similar between groups in each trimester (FIGS. 9B and 9C).

[0094] Given this significant increase in copeptin, we constructed receiver operating characteristic curves for each trimester to interrogate if maternal plasma copeptin concentration was predictive of the development of preeclampsia. Furthermore, optimal copeptin concentration cutoffs were determined from these curves. As seen in FIG. 10, the ROCs demonstrated significant areas under the curve in the first trimester (AUC=0.80, p=0.005), second trimester (AUC=0.87, p=0.002), and third trimester (AUC=0.72, p=0.004). These data indicate that the mean maternal plasma copeptin concentration is predictive of the development of preeclampsia.

[0095] Further, we determined if clinically significant covariates would alter the association of the development of preeclampsia and copeptin concentration at particular trimesters. Logistic regression models were constructed with the diagnosis of preeclampsia as the dependent variable. Participants were dichotomized according to being above or below the determined cutoff for a particular trimester. Using the trimester specific cutoff values (first trimester: 1018 pg/mL, second trimester: 943 pg/mL, third trimester: 860 pg/mL), models were generated using the status of being above or below the cutoff as an independent variable while controlling for clinically significant covariates. After controlling for clinically significant covariates such as maternal age, body mass index, diabetes, chronic hypertension, history of preeclampsia, and twin gestation, copeptin concentration was still significantly associated with the development of preeclampsia in the first, second and third trimester (Table 6). With the exception of the model including the second trimester [copeptin] cutoff and a history of preeclampsia, all models significantly predict the development of preeclampsia. These results confirm our observation that copeptin concentration is significantly elevated in the plasma of pregnant women who will develop preeclampsia in comparison to controls. This robust elevation in copeptin concentration occurs early in the first trimester and remains elevated throughout pregnancy despite potential confounding effects of clinically significant obstetrical and vascular covariates. Finally, we observed that the chronic elevation of vasopressin during pregnancy is sufficient to cause preeclampsia-like phenotypes in mice. Vasopressin infusion significantly reduced the rate of pregnancy (FIG. 11A), highlighting a role for this hormone in reproductive pathophysiology. Vasopressin infusion during successful pregnancy resulted in cardinal preeclampsia phenotypes, including a robust increase in blood pressure and apparent proteinuria (FIG. 11B), substantial fetal growth restriction (FIG. 11C) and pathognomic glomerular endotheliosis (FIG. 11D).

Table 6. Using first, second and third trimester specific cutoffs, maternal plasma copeptin remains significantly predictive of the development of preeclampsia despite adjustment of significant clinical covariates.

First Trimester Model [Copeptin] Cutoff = 1018 pg/mL	**Beta [Copeptin]**	**Adjusted Odds Ratio**	**P Value**
1st Trimester [Copeptin]	1.8	6.05	0.025
1 st Trimester [Copeptin] + Maternal Age	2.2	9.03	0.018
1st Trimester [Copeptin] + Body Mass Index	1.8	6.05	0.026
1st Trimester [Copeptin] + Diabetes	2.1	8.17	0.024
1st Trimester [Copeptin] + Chronic Essential Hypertension	1.9	6.69	0.024
1st Trimester [Copeptin] + History of Preeclampsia	2.6	13.46	0.028
1st Trimester [Copeptin] + Twin Gestation	1.6	4.95	0.05
Second Trimester Model [Copeptin] Cutoff = 943 pg/mL	**Beta [Copeptin]**	**Adjusted Odds Ratio**	**P Value**
2nd Trimester [Copeptin]	2.8	16.44	< 0.001
2nd Trimester [Copeptin] + Maternal Age	3.1	22.20	< 0.001
2nd Trimester [Copeptin] + Body Mass Index	2.9	18.17	< 0.001
2nd Trimester [Copeptin] + Diabetes	2.8	16.44	< 0.001
2nd Trimester [Copeptin] + Chronic Essential Hypertension	3.2	24.53	< 0.001

(continued)

Second Trimester Model [Copeptin] Cutoff = 943 pg/mL	**Beta [Copeptin]**	**Adjusted Odds Ratio**	**P Value**
2nd Trimester [Copeptin] + History of Preeclampsia	20	485165195.41	0.995
2nd Trimester [Copeptin] + Twin Gestation	3.1	22.20	0.0047
Third Trimester Model [Copeptin] Cutoff = 860 pg/mL	**Beta [Copeptin]**	**Adjusted Odds Ratio**	**P Value**
3rd Trimester [Copeptin]	1.3	3.67	0.017
3rd Trimester [Copeptin] + Maternal Age	1.3	3.67	0.017
3rd Trimester [Copeptin] + Body Mass Index	1.4	4.06	0.012
3rd Trimester [Copeptin] + Diabetes	1.7	5.47	0.008
3rd Trimester [Copeptin] + Chronic Essential Hypertension	1.3	3.67	0.017
3rd Trimester [Copeptin] + History of Preeclampsia	1.3	3.67	0.038
3rd Trimester [Copeptin] + Twin Gestation	1.6	4.95	0.008

[0096] Our data demonstrates that copeptin is a strong predictor of the development of preeclampsia. More importantly, it is predictive of the development of preeclampsia throughout pregnancy as early as the sixth gestational week. This finding represents a major advance in the prediction of preeclampsia. Currently, anti-angiogenic factors like sFLT-1 and Endoglin are elevated as early as 12 weeks before the diagnosis of preeclampsia (50). Follow up analyses of sFLT-1, Endoglin, and other anti-angiogenic factors suggest that testing characteristics of these factors are poor in application to clinical practice (44). Furthermore, a limitation of these factors is that the significant changes in antiangiogenic factors overall have been reported to occur only as early as the second trimester.

[0097] In recent years, substantial effort has been invested to identify first trimester predictors of preeclampsia. These investigations have included first trimester circulating hyperglycosylated human chorionic gonadotropin (hCG) (41), Interleukin-1β (78), high sensitivity C-reactive protein (42), and Pregnancy-associated plasma protein-A (PAPPA) (15). These factors have been shown to be poor to moderately predictive of preeclampsia. Given the promise of antiangiogenic markers in the pathogenesis of preeclampsia, they have been investigated in the first trimester. In conjunction with uterine artery Doppler (UAD) analyses, these factors have been shown to only be moderately predictive (AUC=0.74) of preeclampsia (60). An elevated uterine artery Doppler pulsatile index in the first trimester is correlated with the development of preeclampsia. Poon et al. demonstrated that UAD coupled with maternal history and aneuploidy markers in the first trimester can be very predictive of preeclampsia with AUC = 0.96. In and of itself, UADs have an AUC = 0.91 (67, 68). Although this may be a powerful tool, reliable UAD requires substantial training for sonographers to decrease significant interassay variability through verified programs such as the Fetal Medicine Foundation (64). Such training may not be as available in all hospital settings. Clearly, there is utility in finding a simple predictor of preeclampsia as early in pregnancy as possible, and copeptin represents the first simple and individually predictive biomarker. Coupling plasma copeptin measures with other known first-trimester assays may further increase predictive power. Multiple processes involving placental dysregulation, endothelial cell dysfunction, immunology, oxidative stress, altered vascular biology, and angiogenesis make finding a singular cause of preeclampsia nearly impossible. As preeclampsia is a disease resulting from multiple pathways, the development of a predictive model and the search for a therapeutic pathway for preeclampsia treatment may need to come from the upstream regulators or inducers of these multiple pathways. Vasopressin sits at the crux of many of these pathways. The acknowledgement of copeptin, and thereby vasopressin secretion, as a novel, very early-pregnancy diagnostic biomarker for preeclampsia, plus results from our vasopressin-infused mouse model collectively support the hypothesis that elevated vasopressin secretion in early pregnancy may contribute to the development of preeclampsia. Arginine vasopressin is a peptide hormone synthesized primarily within magnocellular neurons of the supraoptic nucleus and paraventricular nuclei of the brain, though it is produced by selected peripheral tissues in small quantities. Axonal projections from magnocellular neurons comprise the posterior pituitary gland, and upon stimulation vasopressin is released into the circulation. Vasopressin then acts upon multiple receptor types to ultimately increase blood volume, vascular constriction, and reduce osmolality (70).

[0098] The connection of vasopressin to the pathogenesis of preeclampsia is strengthened by the immunoactive nature of vasopressin and the immunologic initiating events of preeclampsia. As reviewed by Russell and Walley (70), and by Chikanza and Grossman (12), vasopressin has a variety of immunomodulatory effects. Depending on site of action and dose, vasopressin is known to affect and be affected by tumor necrosis factor-α, interleukin-1β, interferon-γ, β-endorphin, and prostaglandin E2 - many of which are altered in preeclampsia. Vasopressin is known to stimulate the autologous mixed lymphocyte response. Vasopressin is produced by, and acts upon, human T cells, B cells, and monocytes/macrophages. High doses of vasopressin cause an amplification of prostaglandin E2 synthesis by human dermal fibroblasts. Further, vasopressin-deficient hypertension Brattleboro rats exhibit substantial changes in circulating immune cell populations and function, including increased neutrophils. These data suggest a potential link between the elevated vasopressin secretion in early pregnancy observed in the present study with excessive peripheral immune activation, and the subsequent development of preeclampsia. Based on our data and others, we therefore posit that vasopressin may play an important role in initiating the immunologic milieu that precipitates preeclampsia.

[0099] Our study has benefitted from the high quality of clinical data and biosample fidelity provided by the Maternal Fetal Tissue Bank. Furthermore, our study was strengthened by being appropriately powered to evaluate our desired outcomes. One weakness of our study is the predominantly Caucasian population in Iowa. Even though the relationship of copeptin and preeclampsia is robust after clinical covariate adjustment, we are not appropriately powered to analyze the variance due to race. A larger sample size would be necessary for that analysis despite finding significant covariate adjusted associations.

[0100] The temporal organization of molecular events and clinical associations that define preeclampsia has been somewhat muddled to date, as essentially all known mechanisms occur or develop in rapid succession during late-pregnancy. Our data clearly demonstrate an early-pregnancy elevation in vasopressin secretion, thus aligning all other known mechanisms as potential targets of vasopressin action. These results highlight the utility of plasma vasopressin / copeptin measurements in the prediction of preeclampsia, and are consistent with a potential causative role for vasopressin in preeclampsia. While our data from mice demonstrate the sufficiency of vasopressin to cause preeclampsia-like phenotypes, future studies are required to elucidate the tissues, receptors, and mechanisms that mediate the induction of preeclampsia by vasopressin. Substantial clinical studies are required to assess the necessity of vasopressin signaling for the development of preeclampsia, and the utility of targeting this system to treat the disorder. Finally, additional investigations will be required to identify the mechanisms that induce excessive vasopressin secretion, to better understand the event(s) that initiate preeclampsia.

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<400> 28
gggttgttga caaacatcac ctgagc 26

<210> 29
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> B-actin forward

<400> 29
catcctcttc ctccctggag aaga 24

<210> 30
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> B-actin reverse

<400> 30
acaggattcc atacccaaga aggaagg 27

Claims

1. A method of diagnosing or predicting the likelihood of occurrence of preeclampsia in a subject, the method comprising:

   measuring differences in copeptin levels in a sample collected from a subject during the first trimester of pregnancy compared to a control using an antibody detection assay, wherein the sample is blood, serum, plasma or urine, and
   wherein an increase in copeptin levels of about 1/4 fold compared to a control is predictive of the occurrence of preeclampsia during the subject's pregnancy.

2. The method of claim 1, further comprising taking Doppler velocimetry measurements on at least one of the subject's uterine and umbilical arteries.

3. The method of claim 1, wherein the sample is urine.

4. The method of claim 1, wherein the assay comprises a test strip or an ELISA.

Patentansprüche

1. Verfahren zur Diagnose oder Voraussage der Wahrscheinlichkeit des Auftretens von Präeklampsie in einem Lebewesen, wobei das Verfahren umfasst:

   Messen von Unterschieden in Copeptin-Spiegeln in einer Probe, die einem Lebewesen während des ersten Schwangerschaftstrimesters entnommen wurde, im Vergleich zu einer Kontrolle unter Verwendung eines Antikörpernachweis-Assays, wobei die Probe Blut, Serum, Plasma oder Urin ist, und
   wobei ein Anstieg in Copeptin-Spiegeln von etwa 1/4 -fach im Vergleich zu einer Kontrolle voraussagend für das Auftreten von Präeklampsie während der Schwangerschaft des Lebewesens ist.

2. Verfahren nach Anspruch 1, das ferner ein Durchführen von Doppler-Velozimetrie-Messungen an mindestens einer der Gebärmutter- und Nabelarterien des Lebewesens umfasst.

3. Verfahren nach Anspruch 1, wobei die Probe Urin ist.

4. Verfahren nach Anspruch 1, wobei der Assay einen Teststreifen oder einen ELISA umfasst.

Revendications

1. Procédé de diagnostic ou de prédiction de la probabilité d'apparition d'une pré-éclampsie chez un sujet, le procédé comprenant :

   la mesure des différences des taux de copeptine dans un échantillon collecté chez un sujet au cours du premier trimestre de grossesse par comparaison à un contrôle en utilisant une analyse de détection d'anticorps, dans lequel l'échantillon est du sang, du sérum, du plasma ou de l'urine, et
   dans lequel une augmentation des taux de copeptine d'environ ¼ par comparaison à un contrôle est prédictive de l'apparition d'une pré-éclampsie pendant la grossesse du sujet.

2. Procédé selon la revendication 1, comprenant en outre la prise de mesures de vélocimétrie Doppler sur au moins une des artères utérines et ombilicales du sujet.

3. Procédé selon la revendication 1, dans lequel l'échantillon est de l'urine.

4. Procédé selon la revendication 1, dans lequel l'analyse comprend une bandelette de test ou un ELISA.

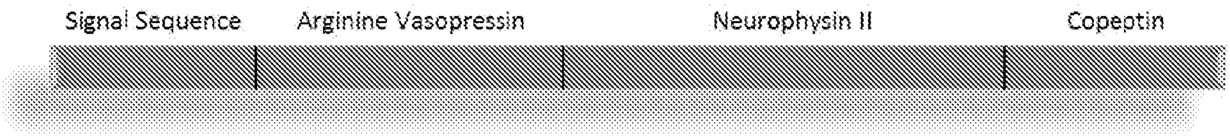
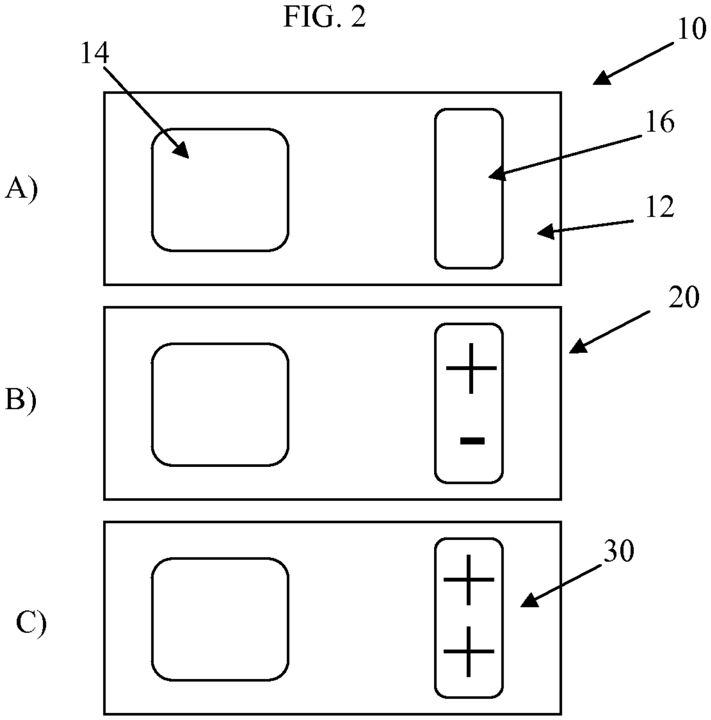

Signal Sequence
Arginine Vasopressin
Neurophysin II
Copeptin

FIG. 1

FIG. 2


10
14
16
12
20
30
A)
B)
C)
+
-
+
+

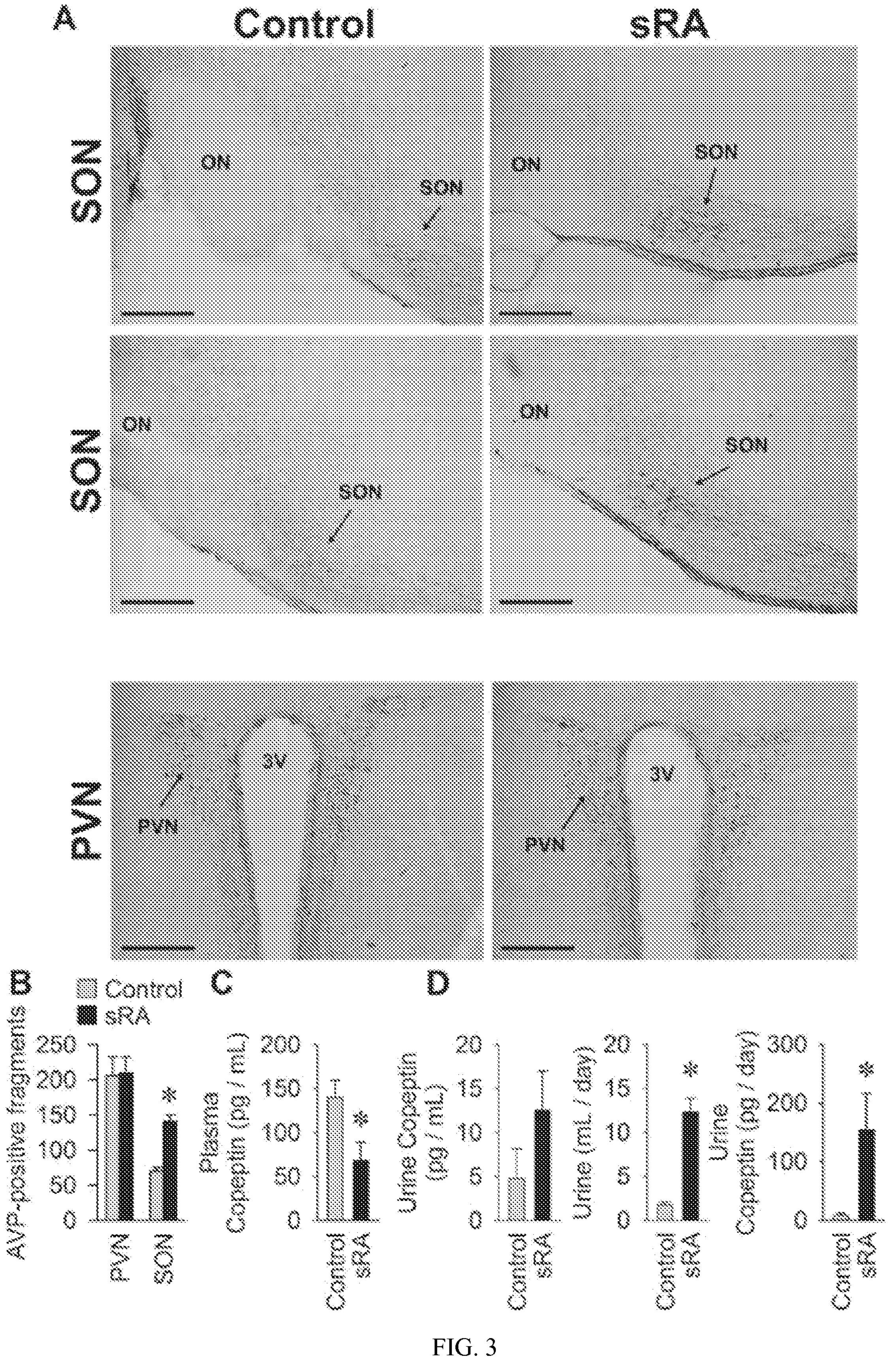

A
Control
sRA
SON
ON
SON
ON
SON
SON
ON
SON
ON
SON
PVN
3V
PVN
3V
PVN
B
Control
sRA
AVP-positive fragments
250
200
150
100
50
0
PVN
SON
*
C
Plasma Copeptin (pg / mL)
200
150
100
50
0
Control
sRA
*
D
Urine Copeptin (pg / mL)
20
15
10
5
0
Control
sRA
Urine (mL / day)
20
15
10
5
0
Control
sRA
*
Urine Copeptin (pg / day)
300
200
100
0
Control
sRA
*

FIG. 3

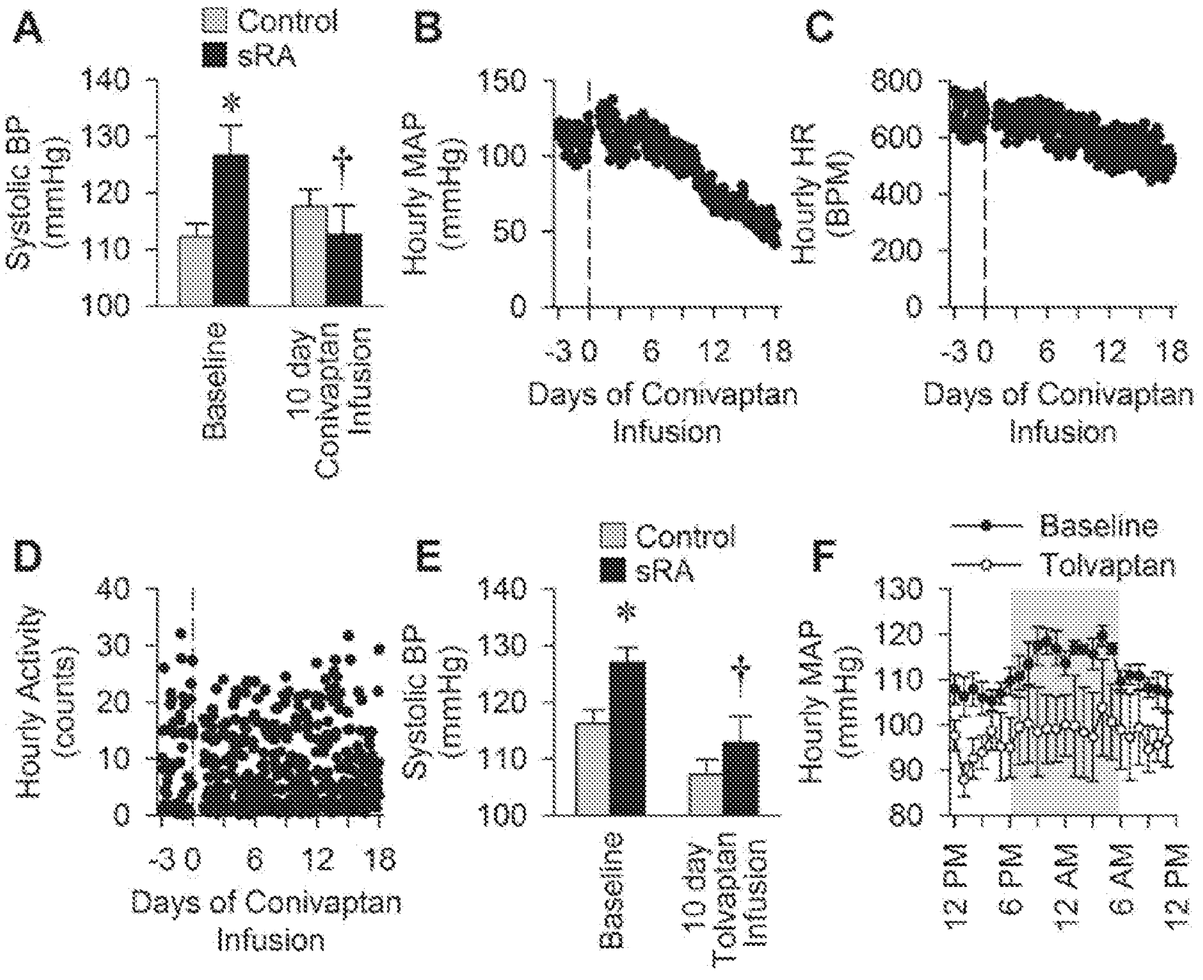

A
▨ Control
■ sRA
Systolic BP (mmHg)
140
130
120
110
100
*
†
Baseline
10 day Conivaptan Infusion
B
Hourly MAP (mmHg)
150
100
50
0
-3 0 6 12 18
Days of Conivaptan Infusion
C
Hourly HR (BPM)
800
600
400
200
0
-3 0 6 12 18
Days of Conivaptan Infusion
D
Hourly Activity (counts)
40
30
20
10
0
-3 0 6 12 18
Days of Conivaptan Infusion
E
▨ Control
■ sRA
Systolic BP (mmHg)
140
130
120
110
100
*
†
Baseline
10 day Tolvaptan Infusion
F
—●— Baseline
—○— Tolvaptan
Hourly MAP (mmHg)
130
120
110
100
90
80
12 PM
6 PM
12 AM
6 AM
12 PM

FIG. 4

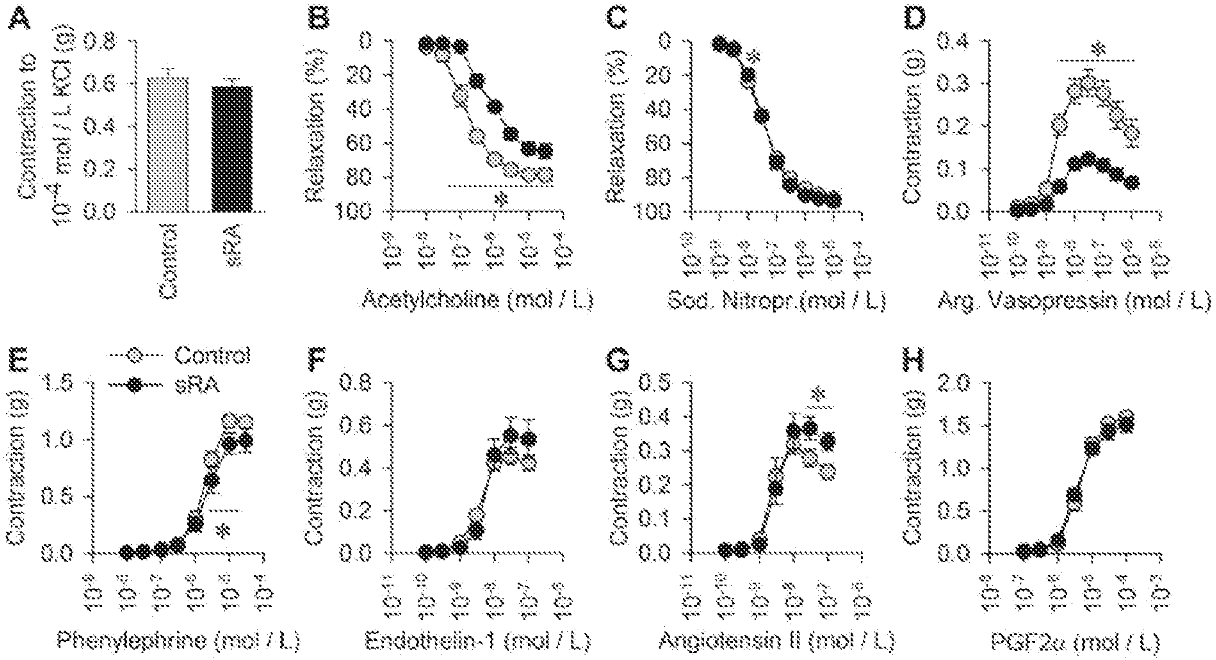

A
Contraction to
10^{-4} mol / L KCl (g)
0.8
0.6
0.4
0.2
0.0
Control
sRA
B
Relaxation (%)
0
20
40
60
80
100
*
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Acetylcholine (mol / L)
C
Relaxation (%)
0
20
40
60
80
100
*
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Sod. Nitropr.(mol / L)
D
Contraction (g)
0.4
0.3
0.2
0.1
0.0
*
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Arg. Vasopressin (mol / L)
E
Control
sRA
Contraction (g)
1.5
1.0
0.5
0.0
*
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Phenylephrine (mol / L)
F
Contraction (g)
0.8
0.6
0.4
0.2
0.0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Endothelin-1 (mol / L)
G
Contraction (g)
0.5
0.4
0.3
0.2
0.1
0.0
*
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Angiotensin II (mol / L)
H
Contraction (g)
2.0
1.5
1.0
0.5
0.0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
PGF2α (mol / L)

FIG. 5

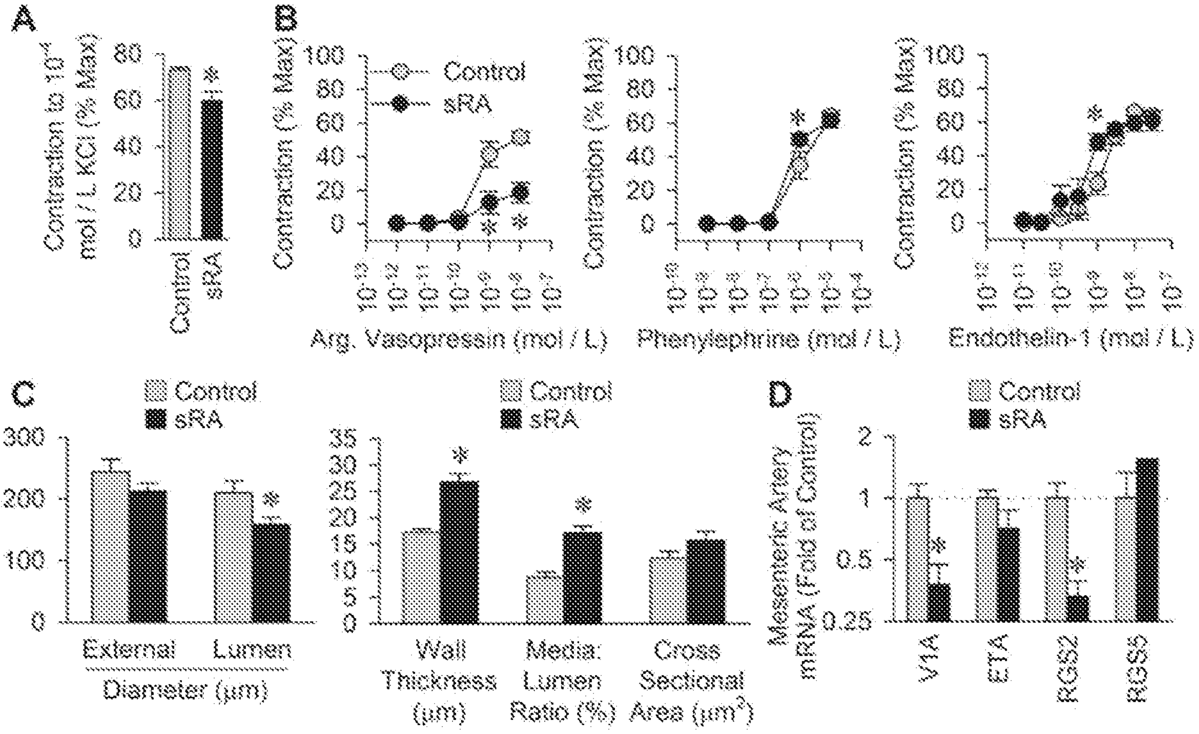

A
Contraction to 10^{-4} mol / L KCl (% Max)
80
60
40
20
0
Control
sRA
*
B
Contraction (% Max)
100
80
60
40
20
0
Control
sRA
*
*
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Arg. Vasopressin (mol / L)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Phenylephrine (mol / L)
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Endothelin-1 (mol / L)
C
Control
sRA
300
200
100
0
External
Lumen
Diameter (μm)
35
30
25
20
15
10
5
0
Wall Thickness (μm)
Media: Lumen Ratio (%)
Cross Sectional Area (μm^2)
D
Mesenteric Artery mRNA (Fold of Control)
2
1
0.5
0.25
V1A
ETA
RGS2
RGS5

FIG. 6

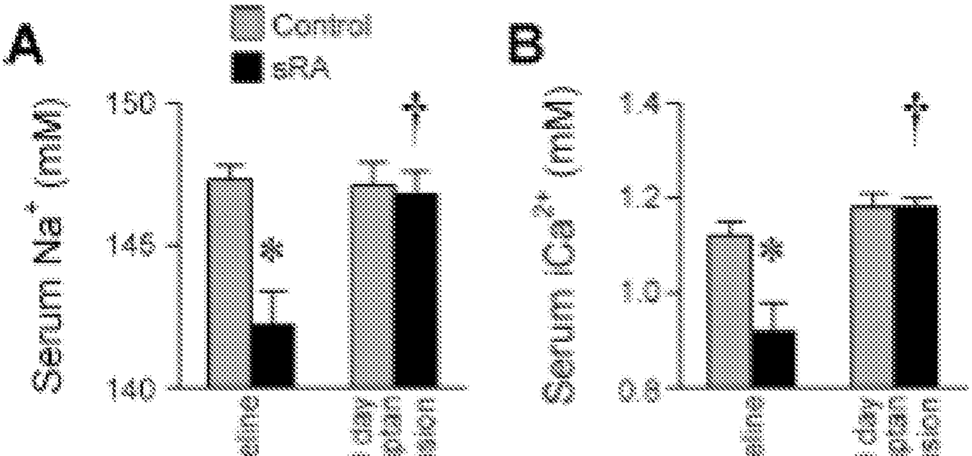

Control
sRA
A
Serum Na^+ (mM)
150
145
140
*
†
Baseline
10 day Tolvaptan infusion
B
Serum iCa^{2+} (mM)
1.4
1.2
1.0
0.8
*
†
Baseline
10 day Tolvaptan infusion

FIG. 7

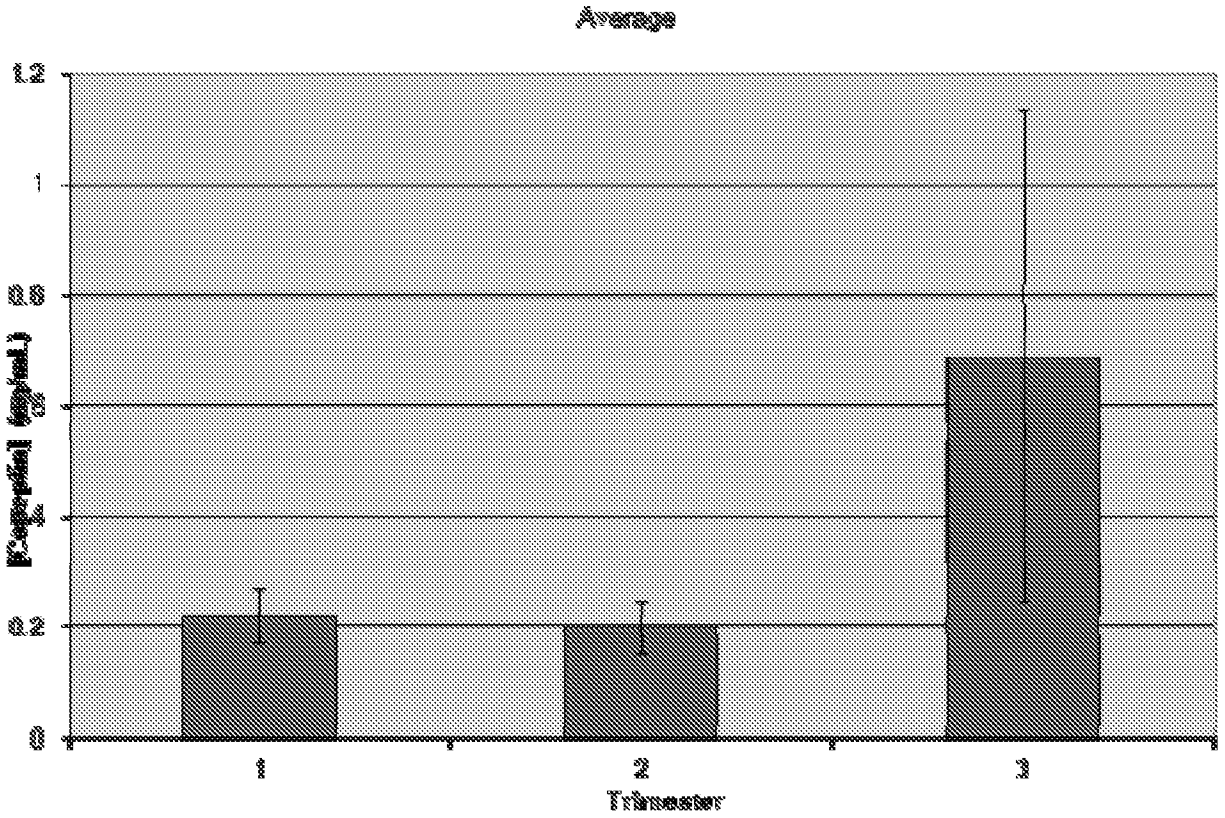
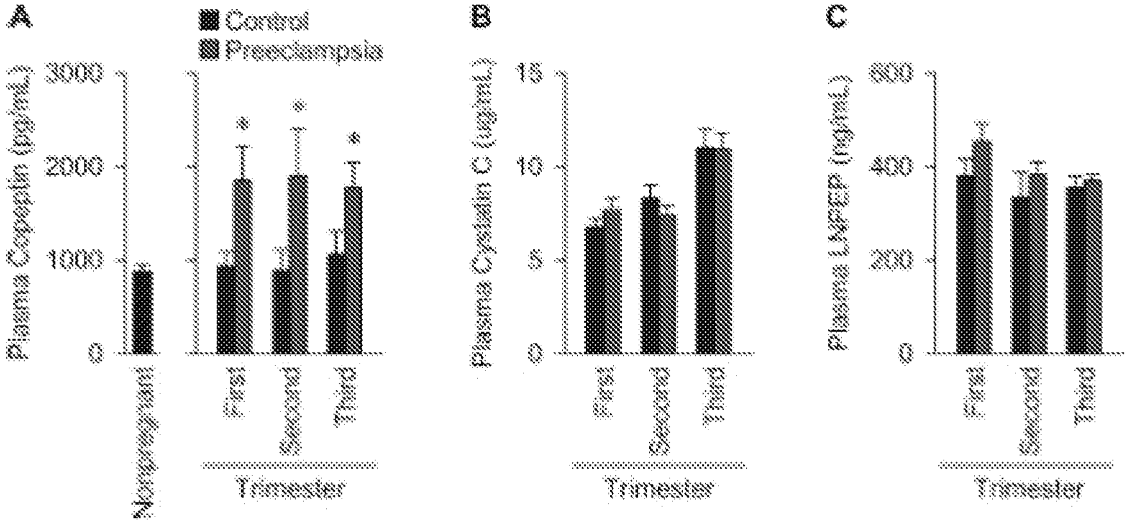
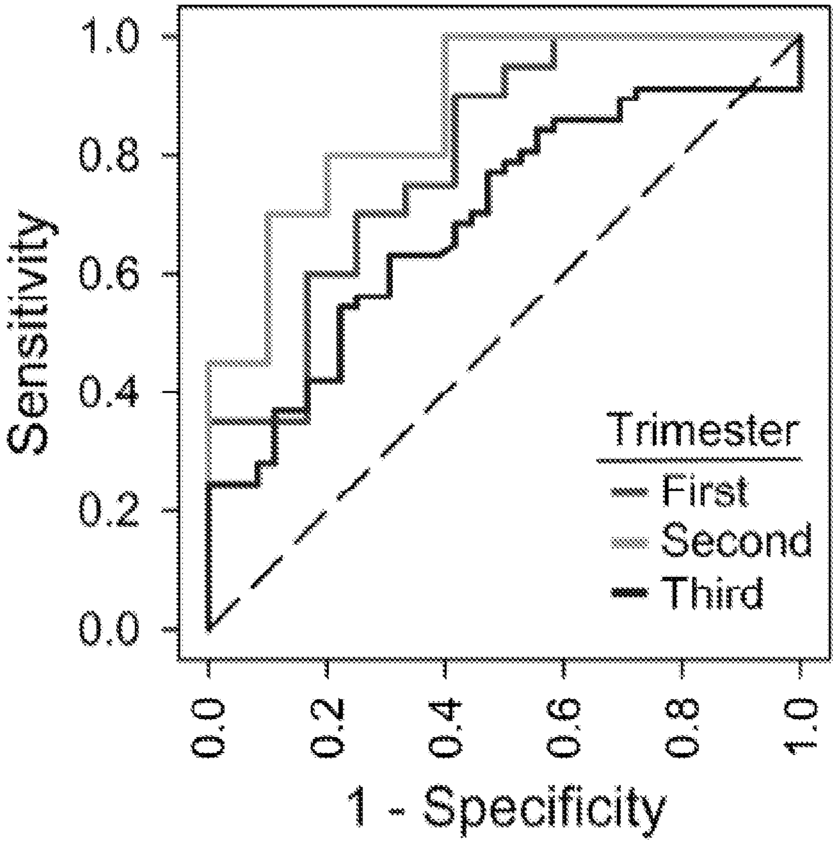


FIG. 8



69x31mm (600 x 600 DPI)

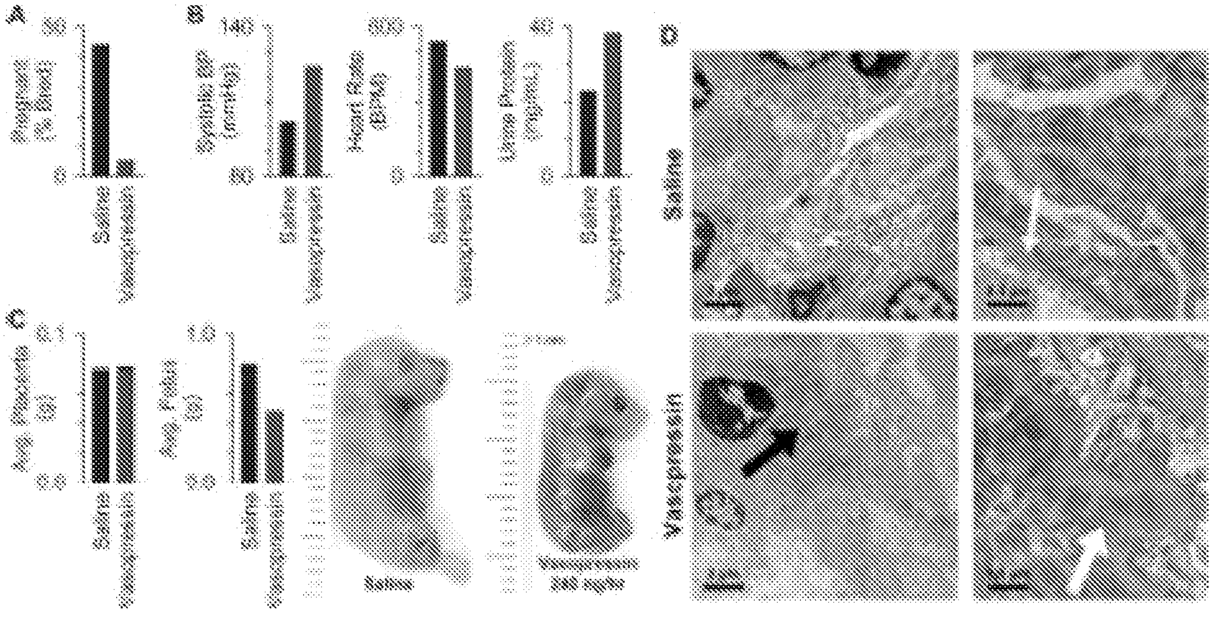
FIG. 9



Trimester	AUC	P Value	Cutoff (pg/mL)	Sensitivity	Specificity
First	0.80	0.005	1018	75%	67%
Second	0.87	0.002	943	82%	78%
Third	0.72	0.004	860	71%	64%

82x90mm (600 x 600 DPI)

FIG. 10



89x44mm (600 x 600 DPI)

FIG. 11

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摘要(译)

公开了用于诊断或预测受试者中先兆子痫发生的可能性的测定，试剂盒，方法和装置。